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Effect of Excipient Gelucire 44/14 on the Intestinal Transport of Diltiazem Hydrochloride in Male Wistar Rat

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ABSTRACT: Excipients play a crucial role in drug disintegration, dissolution, absorption, distribution and transport. They are used in pharmaceutical formulations to modulate drug transport by multiple mechanisms including inhibition of intestinal P-gp. The main aim of this work was to investigate the effects of Gelucire 44/14 on the P-gp-mediated transport of Diltiazem a CYP3A4 substrate across the rat intestine.Diltiazem is mainly used in the treatment of hypertension and angina pectoris. It is mainly metabolized to desacetyl-Diltiazem and desacetyl-N-demethyl-Diltiazem in which CYP3A mediates this N-demethylation and desacetylation. Everted and non-everted sac methods were used to study the transport of Diltiazem across the rat intestine (duodenum, jejunum and ileum). The rats were treated with Gelucire 44/14 using different concentrations (0.5%, 1%).The rats were sacrificed by using anesthetic ether. The intestinal segments were isolated and used for the studies. The probe drug (Diltiazem) solution 10 mg/mL was placed in the isolated intestinal sac and placed in buffer (Dulbecco's). Samples were collected periodically. The drug content was estimated using high performance liquid chromatography method (HPLC).Control experiments were also performed. The transport of Diltiazem increased in case of non everted studies and exsorptionvalues were found to decrease compared to control incase of everted studies. Results reveal that gelucire 44/14 at 1% concentration inhibit P-gp action effectively when compared to gelucire 44/14 at 0.5% concentration.

KEYWORDS: Diltiazem, Gelucire44/14, Drug transport mechanisms, Everted, Non-everted, Exsorption and High Performance Liquid Chromatography method (HPLC).

I. INTRODUCTION

In the global market the role of oral dosage forms is the most common route of administration from decades. Though patient compliance of more the optimized formulation is necessary for better pharmacological action of the drug. Such optimizations can include an improvement of bioavailability, a reduction of side-effects or a decrease in the frequency of administration. Absorption of oral drug in the intestine is an important factor to determine the drug bioavailability. There are many intestinal transporters expressed on the small intestine, they mediate drug absorption, distribution, excretion, and drug-drug interaction. Apart from passive absorption, transporters and pumps mediate active transport thus play an important role in the absorption of nutrients and drugs from the intestine into the blood circulation which shows affinity to intestinal transporters [1].

In order to exert pharmaco dynamic actions, the drug had to pass through the bilipid layers including the transporters embedded in the cell membrane. Among the transporters ABC (ATP-binding cassette superfamily) transporter consists of some subfamilies, P-gp, MRP are particularly considered because they actively remove a wide range of drugs against concentration gradient. ABC transporter is driven by ATP hydrolysis energy which causes conformational changes in the transporter. Carrier-mediated transport processes are saturable and inhibitable, and may be regulated by a variety of external and internal factors. Saturability of carrier-mediated transport may lead to dose-dependent pharmacokinetics of drugs that are substrates of carriers. Induction as well as inhibition of carriers involved in drug transport may lead to diminished or enhanced absorption of drugs with affinity for these carriers [2-3]. In recent years researchers are emphasizing on P-gp inhibitors to improve bioavailability by inhibiting P-gp in intestine, brain, liver, kidneys etc. generally P-gp can be inhibited by blocking drug binding site either competitively, non-competitive or allosterically, interfering ATP hydrolysis and by altering integrity of the cell membrane lipids [4].

Diltiazam is a potent vasodilator which reduces peripheral resistance and after load. It interferes with the movement of calcium into heart muscle cells and the smooth muscle cells in the walls of the arteries. Diltiazem is well absorbed from the gastrointestinal tract. It is distributed throughtout the body. In vitro ligand binding studies show Diltiazem is 70% to 80% bound to plasma proteins mainly hepatic metabolism and is subjected to an extensive first pass effect. The plasma elimination half-life following single or multiple drug

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administration is approximately 3.0 to 4.5 hours and is excreted by urine (2-4% as unchanged and metabolites), bile (remaining metabolites) [5].

II. MATERIALS AND METHODS

Materials

Diltiazem Hydrochloride was obtainedfromDivi's Labs (Hyderabad, India).Verapamil pure drug was gifted from Dr. Reddy's Lab Ltd.0020(Hyderabad, India). Gelucire 44/14 was gifted from Gattfosse,India. Methanol HPLC grade was gifted from E. Merck (India) Limited, Mumbai. Dulbecco's buffer was gifted from Hi Media (India) Limited, Mumbai.Acetonitrile HPLC grade was gifted from Qualigens fine chemicals, Mumbai. Glucose was gifted from Hi Media (India) Limited, Mumbai. Orthophosphoric acid was gifted from E. Merck (India) Limited, Mumbai. Tri ethyl amine was gifted from E. Merck (India) Limited, Mumbai and all chemicals used in this study are AR grade.

Experimental subjects:MaleWistar rats were taken as subjects weighing about 200±25 g [1]. The everted and non-everted study was conducted according to the protocol approved by animal ethics committee; Kakatiya University, India and the studies were conducted in Vaagdevi college research laboratories.

Methods

***Normal sac study*:** Experimental subjects, Male wistar rats were fasted overnight with free access to water before the experiments. Animals were anesthesised with pentobarbital (30 mg/kg/ip) and the whole small intestine was isolated and flushed with 50 ml of ice-cold saline. Intestine was divided into 3 segments of equal length (10 cm) and normal sacs were prepared. Diltiazem (1 mg/mL), was used as probe drug solution was prepared by dissolving in pH 7.4 isotonic Dulbecco's PBS (D-PBS) containing 25 mM glucose. The probe drug solution (1mL) was introduced into the normal sac (mucosal side), and both ends of the sac were ligated tightly. The sac containing probe drug solution was immersed into 40 mL of D-PBS containing 25 mM glucose in the mucosal side. The medium was pre-warmed at 37 ^{0}C and pre-oxygenated with 5% CO_2/ 95% O_2 for 15 minutes, under bubbling with a CO_2/O_2 mixture gas and the transport of the Diltiazem from mucosal to serosal surfaces across the intestine was measured by sampling the serosal medium periodically for 120 minutes [1,6].

***Everted sac study*:**Evertdsac study was done with Male Wister rats which were fasted overnight with free access to water before the experiments and anesthetized with pentobarbital (30 mg/kg/ip) were exsanguinated, and the whole small intestine was isolated and flushed with 50 mL of ice-cold saline. It was later divided into 3 segments of equal length (10 cm) and each segment was everted. Diltiazem (1mg/mL), was used as probe drug solution was prepared by dissolving in pH 7.4 isotonic Dulbecco's PBS (D-PBS) containing 25 mm glucose [7-9]. The probe drug solution (1mL) was introduced into the everted sac (serosal side) and both ends of the sac were ligated tightly. The sac containing probe drug solution was immersed into 40 mL of D-PBS containing 25 mm glucose in the mucosal side. The medium was pre-warmed at 37 0C and pre-oxygenated with 5% CO2/ 95% O2 for 15 minutes, under bubbling with a CO2/O2 mixture gas and the transport of the Diltiazem from serosal to mucosal surfaces across the intestine was measured by sampling the mucosal medium periodically for 120 minutes [2].

***Inhibition studies*:**In inhibition study, inhibitor Verapamil 250µm was added to mucosal medium. Using these media the transport of Diltiazem in absence (control) or presence of inhibitor (250µm Verapamil) and under induced conditions after treatment with Gelucire 44/14 in different ratios(0.5 and 1mg/kg/po) the efficiency was studied.

***Ex-vivo transport study*:**The transport of Diltiazem across rat intestine (duodenum, jejunum, and ileum) was studied by using Ex-vivo everted and non-everted sac methods. The rats were treated separately with gelucire 44/14 (0.25,0.5, 1mg/kg) and Verapamil (1mg/kg) in three groups for 7 days sacrificed, the intestinal segments were isolated and then the sac were prepared. The drug solution was placed in sac and kept in 40mL dulbecco's buffer. Samples were collected at preset time point for 120min by replacing with fresh buffer and their drug content were estimated using validated HPLC method. Control experiments were also performed [10].

***Precipitation method*:** 100µL Methanol was added to intestinal sac samples (200µL) vortexed for two minutes and centrifuged (heraeus, Germany) at 2500 rpm for 15 min. The supernatant (20µL) was injected into HPLC column with the help of Hamilton syringe [1].

***HPLC Analysis*:**The Shimadzu High Performance Liquid Chromatography system equipped with a LC-20AT pump and SPD 10 AT UV visible detector and RP C18 column (kromasil, 250mm x 4.6 mm ID and particle size 5µm) was used for the analysis of samples. The

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mobile phase used was a mixture of acetonitrile, potassium phosphate buffer (10mM, pH 4.6) 35:65 v/v, the pH was adjusted to 4.6 with orthophosphoric acid. The elution was monitored at 238nm, at a flow rate of mL/min.

Chromatographic Conditions:

Mobile phase : Double distilled water: Acetonitrile (60:40) v/v.
Pressure : 15.6 M Pa
Column temperature : Room temperature
Flow rate : 1mL/min
UV-detection at : 238nm
Detector sensitivity : 0.0010AUFS
Injection volume : 20µL
Retention time : 6 min

Statistical Analysis: The efflux results were tested for statistical significance using t-test. The difference in the sample means were considered significant at $p<0.05$.

III. RESULTS AND DISSCUSION

Formulations: In the formulations, Drug polymer; ratio 1:1.5, 1:2 were used to study their effect on release of drug from the capsule (Table 1).

Table 1: Formulation preparation by using different concentration of carrier.

Formulation code	Drug:carrier ratio	Drug(mg)	Gelucire 44/14
F1	1:1.	10	10
F2	1:1.5	10	15
F3	1:2	10	20

In the present study, the mean transport of Diltiazem from mucosal to serosal (normal sac and everted sac) was determined in duodenum, jejunum and ileum regions of rat intestine in the absence and presence of Gelucire 44/14. The time course of Diltiazem transport at different concentrations across rat small intestine of duodenum, jejunum and ileum was shown in Table (2&3)

Table 2: Mean ± S.D (n=3) cumulative efflux concentrations (µg/mL) in intestinal Noneverted sacs in male Wistar Rats.

Groups Region	Control	Diltiazem+Gelucire44/14 (Different Concentrations mcg/ml)		Standard
		0.5%	1%	
Duodenm	8.98 ± 1.28	9.4±0.16	12.26±0.30	11.88 ± 1.65
Jejunum	5.04 ± 3.45	5.93±0.38	10.11±0.18	9.30 ± 5.29
Ileum	1.36 ± 0.70	1.5± 0.37	12.3± 0.42	2.72 ± 0.74

Note: statistically significant ($P<0.05$)

Table 3: Mean± S.D (n=3) cumulative efflux concentrations (µg/ml) in intestinal everted sacs in male Wistar Rats.

Region	Control	Diltiazem+Gelucire 44/14 (Different Concentrations mcg/ml)		Standard
		0.5%	1%	
Duodenum	32.78± 1.47	3.01± 0.008	1.54 ± 0.21	16.65± 1.08
Jejunum	15.82 ± 1.18	2.03 ± 0.18	0.86 ±0.06	14.02± 1.15
Ileum	15.81 ± 1.78	2.53± 0.23	0.75± 0.27	12.71± 3.37

Note: Groups statistically significant ($P<0.05$).

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Results of this study reveal that the transport of Diltiazem in non-everted and everted sac methods was reduced by the treatment of Gelucire 44/14, in comparison to the control. There was a statistically significant ($P<0.05$) difference. The transport was increased from duodenum to ileum. The results suggest that both Gelucire 44/14 and Verapamil treatment influences the transport of Diltiazem. As Diltiazem is the substrate for CYP3A, therefore the transport of Diltiazem was increased [11].

The non-everted sac model was originally used to evaluate drug transport mechanisms. Yulian LIN [5]compared the permeability values of some actively transported molecules and passively absorbed compounds through evertedand non-everted sacs and found that the permeability was higher when the sacs were everted. The permeability of passive absorption of drug Diltiazem remained the same whether the sacs were everted or not. These results suggested that the passive permeability of actively transported molecules can be determined through non-everted rat gut sacs.

In everted sac studies exsorption values of Diltiazem decrease from 32.78±1.47 to 3.01±0.008µg in duodenum; 15.82±1.18 to 2.03± 0.18µg in jejunum and 15.81±1.78 to 2.53±0.23µg in ileum with Gelucire 0.5% concentration and the exsorption values of Diltiazem decreased from 32.78±1.47 to 1.54±0.21µg in duodenum; 15.82±1.18 to 0.86±0.06µg in jejunum and 15.81±1.78 to 0.75±0.27µg in ileum with Gelucire 1% concentration With Verapamil there was decreased exsorption values of Diltiazem from 32.78±1.47 to 16.65±1.08µg in duodenum; 15.82±1.18 to 14.02±1.15µg in jejunum an15.81±1.78 to 12.71±3.37µg in ileum (figures 3a, 3b, 3c) .

In non everted sac studies,Gelucire 44/14 treatment increased the mean cumulative amount of Diltiazem from 8.98±1.28 to 9.4±0.16mcg in duodenum; 5.04± 3.45 to 5.93± 0.38µg in jejunum and 1.36±0.72 to 1.5±0.37µg in ileum with Gelucire 0.5% concentration and the mean cumulative amount of Diltiazem decreased from 8.98±1.28 to 12.26±0.30µg in duodenum; 5.04±3.45 to 10.11±0.18µg in jejunum and 1.36±0.72 to 12.35±0.42 with Gelucire 1% concentration. With Verapamil there was an increased cumulative amount of Diltiazem from 8.98±1.28 to 11.88±1.65µg in duodenum; 5.05±3.45 to 9.30±5.29µg in jejunum and 1.36±0.70 to 2.72±0.74µg in ileum (figures 4a, 4b, 4c).

HPLC Studies:

From the figure 2 it can be noticed that drug peak was observed at 6 minutes which was not observed in blank chromatogram in figure 1.

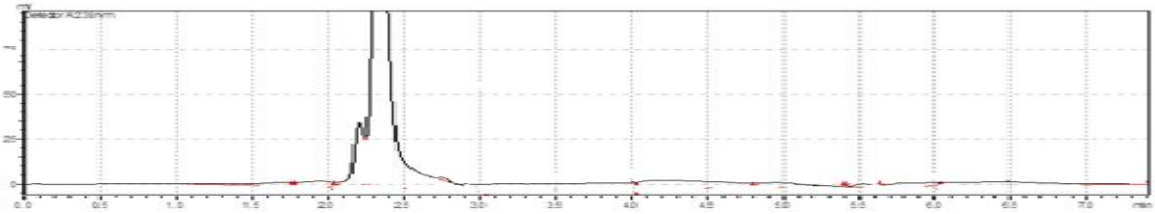

Figure1: HPLC blank Chromatogram.

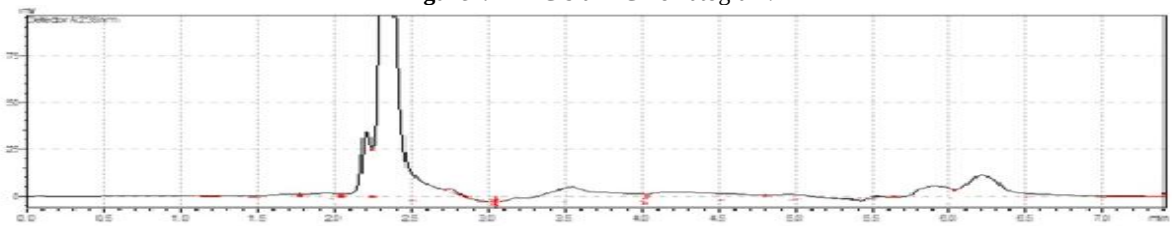

Figure2: HPLC chromatogram of intestinal sac sample.

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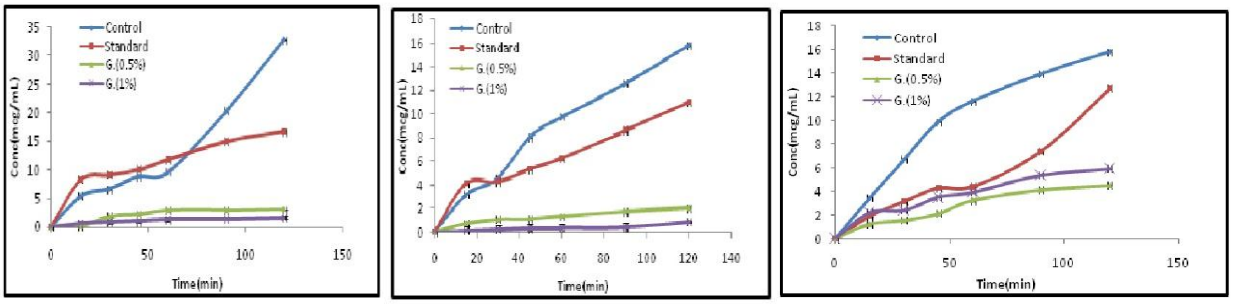

(a) (b) (c)

Figure3: Cumulative transport of Diltiazem in (a) Duodenal, (b)jejunial, (c)IlealEverted sac in Wistar rats.

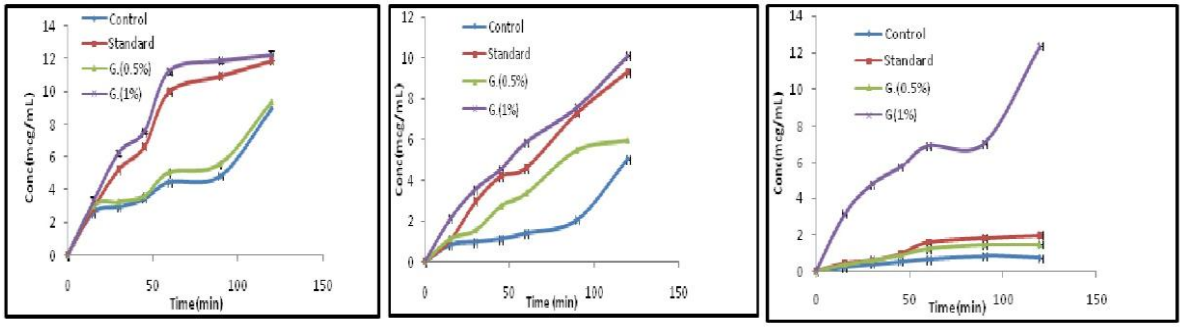

Figure4: Cumulative transport of Diltiazem in (a) Duodenal, (b) jejunial, (c) IlealNon-Everted sac in Wistar rats.

Standard graph of Diltiazem hydrochloride

Standard graph of Diltiazem hydrochloride was constructed using 6.8 pH phosphate buffer (Figure 5). Various concentrations 2 to16 µg/mL were prepared (Table 4). The absorbance of prepared concentrations was measured at 238 nm by adjusting to zero with blank sample. A graph was plotted by taking concentration on x-axis and absorbance on y-axis and best fit line was drawn and regression value and equation was calculated.

Concentration(ug/mL)	Absorbance
0	0
2	0.109
4	0.21
6	0.309
8	0.406
10	0.514
12	0.624
14	0.739
16	0.846

Table 4: Standard graph of Diltiazem

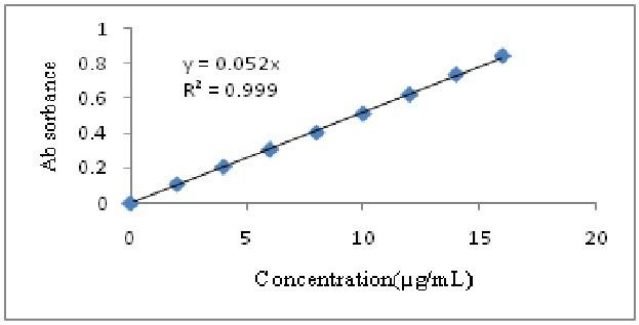

Figure 5: Standard graph of Diltiazem Hydrochloride in 6.8 pH hydrochloride in 6.8 phosphate buffer.buffer*In vitro* Studies of Diltiazem hydrochloride Capsule.

The study was planned to investigate whether transport of Diltiazem at intestinal level is influenced by Gelucire in rat everted and non everted sacs of duodenum, jejunum, and ileum. . Duodenum has less P-gp and more CYP enzymes. Jejenum has moderate P-gp and CYP enzymes. Results of *in vitro* study revealed that Diltiazem transport across the small intestine is affected by P-gp inhibitors.

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Table 5: *In vitro* dissolution profile of formulation F1, F2 and F3 containing Gelucire 44/14

Time (min)	Cumulative % drug release of formulation(n=3)		
	F1	F2	F3
10	33.53±0.13	43.13±2.0	34.40±0.17
15	54.09±0.12	67.54±0.83	58.50±1.50
20	64.36±1.18	80.30±1.25	68.50±0.86
25	72.08±0.94	88.35±0.30	76.56±0.11
30	77.18±0.27	94.03±1.32	82.66±1.04
35	82.84±0.30	97.18±0.32	88.00±0.86
40	87.54±0.50	98.36±0.15	90.53±0.83
45	90.33±0.29	99.28±0.41	91.53±0.50

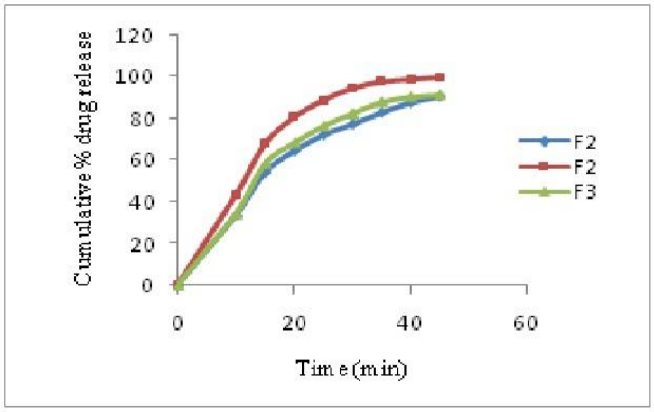

Figure6: *In vitro* release patterns of formulation F1, F2, F3

In Non everted study, the transport of Diltiazem from mucosal to serosal was determined at different anatomic regions of rat small intestine. The transport of Diltiazem increased with time and a greater transport was observed in the ileum.The transport of Diltiazem was increased 1.05, 1.18, 1.2 times after treatment with gelucire 0.5% concentration.The transport of Diltiazem was increased 1.35, 2.18, 9.1 times after treatment with gelucire 1% concentration compared to respective control. As the transport increased it indicates the decreased metabolism by CYP 3A enzyme, therefore the direct inhibition of enzyme by gelucire 44/14 [12-14].

In everted study, the transport of Diltiazem from serosal to mucosal was determined. Exsorption concentrations of Diltiazem was decreased 0.1, 0.13, 0.16 times after treatment with gelucire 0.5% concentration. The exsorption of Diltiazem was decreased 0.05, 0.054, 0.048 times after treatment with gelucire 1% concentration compared to respective control. As the exsorption decreases, absorption increases thereby indicating the indirect inhibition of P-gp by gelucire 44/14.

This observation indicated the role of P-gp, an efflux pump on Diltiazem absorption. Duodenum has less P-gp and more CYP enzymes. Jejenum has moderate P-gp and CYP enzymes. And ileum has more P-gp and less CYP enzymes.

In this present study reveals that 1% concentration of Gelucire 44/14 which effectively inhibits P-gp action on Diltiazem transport in rat intestine.

The study reveals that everted and non-everted sac studies in rat have clearly addressed that the alteration in the oral transport could be due to changes at the absorption site (intestine). There is no linear relationship between everted and non-everted sac methods.

In the preparation of soft cap of gelucire 44/14 the results reveal that increased drug release was observed in dissolution study (Table 5)(Figure 7). And result in enchancing the bioavailability of drug by altering membrane fluidity, and also down regulate P-glycoprotein expression.

Diltiazem has poor bioavailabilty due to extensive first pass metabolism and yields desacetylDiltiazem. Gelucire treatment appears to have significant influence on CYP3A4 mediated intestinal metabolism of Diltiazem. However, it is difficult to extrapolate our results which were obtained in rats to humans. Evaluation of Diltiazem – gelucire 44/14 interaction in humans needs to be verified.

IV CONCLUSION

Treatment with Gelucire 44/14 decreased drug exsorption in everted studies compared to control and increased transport in non everted studies compared to control and indicating the inhibition of P-gp transporter and CYP3A enzyme. Among the different concentrations of gelucire 44/14, 1% was found to inhibit the transporter and enzyme effectively.

The formulation containing Gelucire 44/14 may increase the bioavailabilty of Diltiazem by inhibiting P-gp and CYP 3A enzyme.

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