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Nano-encapsulation of azole antifungals: Potential applications to improve oral drug delivery

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Abstract

The present study was designed to improve the oral bioavailability of two clinically important antifungal drugs—clotrimazole and econazole. Each drug was encapsulated in nanoparticles of a synthetic polymer (polylactide-co-glycolide, PLG) or a natural polymer (alginate stabilized with chitosan). The nanoparticles were prepared by the emulsion–solvent-evaporation technique in case of PLG and by the cation-induced controlled gelification in case of alginate. Drug encapsulation efficiency was better (>90%) for the alginate formulation compared with the PLG formulation (nearly 50%). The formulations were orally administered to mice and the drugs were analyzed in plasma by a validated HPLC technique. The biodistribution/pharmacokinetic data suggested that there was a controlled drug release for 5–6 days with each of the formulations, compared with unencapsulated drugs, which were cleared within 3–4 h of oral/intravenous administration. There was a striking improvement in the relative and absolute bioavailability of each drug. Further, the drugs were detected in the tissues (lungs, liver and spleen) till 6–8 days in case of nanoparticles whereas free drugs were cleared by 12 h. Overall, the alginate formulation appeared to be better than the PLG formulation. The results emphasize the power of nanotechnology to make the concept of enhancement in oral bioavailability of azole antifungal drugs come to reality.

Keywords: Nanoparticles; Clotrimazole; Econazole; Polylactide-co-glycolide; Alginate

1. Introduction

Polymeric nanoparticles have been extensively researched and well established as useful drug carriers. The oral administration of unencapsulated or free drugs often results in a low systemic bioavailability which is mainly attributable to the premature degradation and/or poor solubility of drugs in the gastrointestinal tract, e.g. as in case of some azole antifungal drugs (Fromtling, 1988). The advantages of nano-encapsulation include the enhanced stability of labile drugs, controlled drug release and an enhanced drug bioavailability owing

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to the fact that particles in the nano-size range are efficient in crossing permeability barriers (Jiao et al., 2002; Pandey and Khuller, 2004a). Thus, the absorption and oral bioavailability of heparin (Jiao et al., 2002), enalaprilat (Ahlin et al., 2002), tobramycin (Cavalli et al., 2003) and antitubercular drugs (Pandey et al., 2003) was demonstrated to be significantly enhanced when administered as nanoparticles, in comparison to oral-free drugs. Because of their biodegradability, biocompatibility and approval by US FDA for human use, polylactide-co-glycolide (PLG, a synthetic polymer) and alginate (a natural polymer) are amongst the most popular carriers employed for the purpose of drug encapsulation (Tonnesen and Karlsen, 2002; Bala et al., 2004). The statement is supported by the fact that a number of drugs ranging from anticoagulants (Jiao et al., 2002), antihypertensives (Ahlin et al., 2002), corticosteroids (Horisawa et al., 2002), anti-cancer drugs (Rajaonarivony et al., 1993) and antibiotics (Pandey et al., 2003) have been reported to be successfully incorporated in PLG or alginate nanoparticles. Although polymeric nanoparticles offer the flexibility of being administered through various routes (Pandey et al., 2003a,b; Pandey and Khuller, 2004b), it is the oral route that remains the most preferred one. Keeping in mind that bioavailability is a staggering problem for azole antifungal drugs (only few azoles such as fluconazole show good oral bioavailability) (Fromtling, 1988), the present study was designed at improving the same, by formulating PLG and alginate nanoparticle-based delivery systems. Two azole drugs, i.e. clotrimazole and econazole were selected and the results are being communicated here.

2. Materials and methods

2.1. Chemicals and drugs

Sodium alginate (medium viscosity, 3500 cps for a 2% (w/v) solution), chitosan (minimum 85% deacetylated), clotrimazole, econazole and polyvinyl alcohol (PVA, M_r 13,000–19,000, 85% hydrolyzed) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Polylactide-co-glycolide (50:50 resomer, RG 506, M_r 97,400) was purchased from Boehringer Ingelheim, Germany. HPLC grade solvents and water were obtained from Merck Ltd., Mumbai, India. All other chemicals used in the study were of analytical grade.

2.2. Animals

Laca mice of either sex weighing 20–25 g and obtained from the Central Animal House, Post Graduate Institute of Medical Education and Research, Chandigarh (India) were used in the study. The animals were housed in biosafety cabinets (Nuaire Instruments, NU 605-600E, Series 6) and provided with pellet diet/water ad libitum. The study was approved by the Institute's Animal Ethics Committee.

2.3. Preparation of drug-loaded PLG nanoparticles

Drug-loaded PLG nanoparticles were prepared by the multiple emulsion and solvent evaporation technique (Lamprecht et al., 1999) as described before for other antibiotics (Pandey et al., 2003a). Briefly, distilled water was added to dichloromethane (DCM) containing PLG and either clotrimazole or econazole (drug:polymer::1:1 (w/w); water:DCM::1:10 (v/v)). The mixture was sonicated for 1 min to form the primary emulsion which was poured into 1% (w/v) aqueous PVA and re-sonicated for 3 min. The secondary emulsion so formed was stirred overnight for the removal of DCM, centrifuged (8000–10,000 rpm for 15 min) to harvest the nanoparticles, which were washed with distilled water and vacuum-dried.

2.4. Preparation of drug-loaded alginate nanoparticles

Alginate nanoparticles were prepared by the principle involving cation-induced controlled gelification of alginate (Rajaonarivony et al., 1993). Briefly, calcium chloride (0.5 ml, 18 mM) was added to 9.5 ml of sodium alginate solution (0.06%, w/v) containing either clotrimazole or econazole. The initial ratio of drug: polymer was 7.5:1. Two milliliter of chitosan solution (0.05%, w/v) was added followed by stirring for 30 min and the mixture was kept at room temperature overnight. Drug-loaded nanoparticles were recovered by centrifugation at 19,000 rpm for 30–45 min and

washed thrice with distilled water to obtain the final pellet.

2.5. Characterization of drug-loaded PLG or alginate nanoparticles

The determination of particle size and polydispersity index (PI) was carried out on a Zetasizer 1000 HS (Malvern instruments, UK) based on photon correlation spectroscopy. The drug encapsulation efficiency was determined by the formula: 100—[amount of drug (mg) remaining in the supernatant and pellet washings × 100/amount of drug (mg) initially taken to prepare the nanoparticles], expressed as percent. In vitro drug release studies were carried out in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8) prepared according to the United States Pharmacopoeia (USP 26/NF 21, 2003) at 37 °C under shaking conditions. Aliquots were drawn at 5, 15 and 30 min and 1, 1.5, 2, 2.5, 3, 6, 12, 24 and 48 h, and replaced with equal volume of buffer. The cumulative drug released was calculated and expressed as a percent of the theoretical drug content. The drugs were analyzed by an HPLC system comprising of a dual-piston reciprocating pump, an online de-gasser, a UV–vis dual wavelength detector (each of Series 200) and a 600 Series Link Interface for data acquisition/processing, all from Perkin-Elmer Instruments LLC (Shelton, CT, USA). The drugs were analyzed by employing a USP isocratic program (USP 24/NF 18, 2000) with dibasic potassium phosphate buffer/methanol (1:9, v/v) as the mobile phase (at 1.5 ml/min), 254 nm as the detection wavelength and reversed phase C18 column (Cosmosil 5C18- MS-II from Waters; 250 × 4.6 mm; 5 μm particle size). The method was found to work equally well for clotrimazole and econazole (analytical sensitivity = 0.2 μg/ml for each drug).

2.6. Preparation of drug doses

The drug doses used throughout the study were—clotrimazole 4 mg/kg and econazole 3.3 mg/kg body weight. Because the dose was different for each drug, the initial amount of drug taken to prepare the formulations was calculated by the formula previously described (Pandey and Khuller, 2004c)—(actual amount of drug required × 100/mean drug encapsulation efficiency).

2.7. In vivo drug disposition studies

For the single dose drug disposition studies, mice were grouped as follows: Group 1, intravenous (i.v.) free clotrimazole; Group 2, oral-free clotrimazole; Group 3, oral clotrimazole-loaded PLG nanoparticles; Group 4, oral clotrimazole-loaded alginate nanoparticles; Group 5, i.v.-free econazole; Group 6, oral-free econazole; Group 7, oral econazole-loaded PLG nanoparticles; Group 8, oral econazole-loaded alginate nanoparticles. For each animal, the formulation was suspended in 50 μL distilled water just before oral dosing in each experiment. Free drugs were freshly dissolved in 50 μL methanol/distilled water (1/4, v/v) immediately before dosing. The animals were bled at several time points; 0 (instantaneous), 0.08, 0.25, 1, 2, 3 and 4 h in case of i.v.-free drugs, and 30 and 45 min and 1, 1.5, 2, 2.5, 3 and 6 h, and day 1–7 in case of oral-free drugs/drug-loaded nanoparticles. The plasma obtained from each mouse was deproteinized with methanol (1/1, v/v), vortexed and centrifuged at 5000 × *g* for 20 min at 4–8 °C. The supernatant was used for the analysis of clotrimazole/econazole. The drugs were analyzed by a modification of the HPLC technique discussed above (USP 24/NF 18, 2000) to obtain the plasma drug concentration versus time profile. The method was validated as under:

2.7.1. Linearity

Separate stock solutions of clotrimazole and econazole were prepared in methanol (1mg/ml). Dilutions were made in methanol to give a working range of 0.2–25 μg/ml and analyzed on HPLC. Pooled blank mice plasma was spiked with the drugs separately to give a similar working range. Protein-free filtrate was prepared as above and analyzed by HPLC, keeping the injection volume constant (20 μl). The area obtained at each concentration was compared with the area of the drug in simple methanol to calculate the percentage recovery of the drug. The studies were performed three days in succession.

2.7.2. Accuracy

Known concentration (1 μg/ml) of each drug prepared in blank plasma was analyzed separately. Subsequently, a separate set of the standard solutions was fortified with drug equivalent to 1 μg/ml (low concentration) or 10 μg/ml (high concentration) and analyzed

after appropriate dilutions. For each determination, the percentage recovery was calculated from the difference between the two results.

2.7.3. Precision

Two methods were used to validate precision. In the first, a protein-free filtrate of blank mice plasma containing 1 μg/ml of clotrimazole/econazole was injected five times. In the second, a similar protein-free filtrate was prepared five times separately and each was analyzed. The experiments were performed on three consecutive days and three times on each day.

The area under plasma drug concentration over time curve (AUC_{0-t}) was calculated by the Sigma Plot software (version 8.0) and further used to compute the relative/absolute bioavailability of azoles:

$$\text{Relative bioavailability} = \left(\frac{\text{AUC}_{0\text{-}t} \text{ of oral-encapsulated drugs}}{\text{AUC}_{0\text{-}t} \text{ of oral-free drugs}}\right) \times \left(\frac{\text{dose of oral-free drugs}}{\text{dose of oral-encapsulated drugs}}\right)$$

$$\text{Absolute bioavailability} = \left(\frac{\text{AUC}_{0\text{-}t} \text{ of oral-encapsulated drugs}}{\text{AUC}_{0\text{-}t} \text{ of i.v.-free drugs}}\right) \times \left(\frac{\text{dose of i.v.-free drugs}}{\text{dose of oral-encapsulated drugs}}\right)$$

The animals were sacrificed at different time points (day 1, 3, 6, 7 and 8). In order to validate the analytical technique in case of tissues, liver homogenate was prepared in isotonic saline by removing the liver from a healthy mouse (who had not been administered any drug) and divided into two parts. Each part was further sub divided and spiked with varying concentrations of either clotrimazole or econazole. The protein-free filtrates were analyzed for their drug content by the above-mentioned HPLC techniques and validated for linearity, accuracy and precision. Subsequently, drug levels were determined in 20% (w/v) of tissue homogenates (lungs, liver, spleen, small intestine and kidneys) of drug-treated mice by following the same analytical procedure as described for plasma.

2.8. Statistical analysis

The drug encapsulation efficiency and bioavailability data was analyzed by Student's unpaired *t*-test.

3. Results and discussion

The mean particle size was 217 nm (PI = 0.38) in case of PLG nanoparticles and 235 nm (PI = 0.44) in case of alginate nanoparticles. Both the formulations exhibited a favorable particle size because as far as oral drug delivery is concerned, particles less than 500 nm are known to be taken up intact via transcellular/paracellular pathways in the intestine (Jiao et al., 2002). The drug encapsulation efficiency was better ($p < 0.001$) for the alginate formulation (clotrimazole = 90 ± 3%, econazole = 95 ± 2.5%) compared with the PLG formulation (clotrimazole = 48.33 ± 5.21%, econazole = 52.27 ± 3.80%). Thus, the alginate formulation proved to be more economical because—(i) it could conserve more of the initial drug amount; (ii) cost wise alginate is cheaper than PLG; (iii) on a weight by weight basis, 7.5-fold less amount of alginate was consumed compared with PLG for entrapping equivalent amount of drugs. The in vitro studies indicated that the drug release was low (<9% for the PLG formulation and <3% for alginate formulation), in either SGF or SIF indicating the stability of the formulations.

The methodology employed for the determination of drug encapsulation efficiency as discussed above was tried for drug analysis in mice plasma as well. However, the peaks were not sharp as observed on several occasions. Keeping all other parameters constant, a variation in the composition of mobile phase (methanol/phosphate buffer) from 3/1 to 9/1 (v/v) served to rectify the problem. The analytical technique for each azole drug in plasma was validated for linearity, accuracy and precision of determination (Table 1). The regression equations and correlation coefficients showed that the detector response was strictly linear for each drug in the specified concentration range of 0.2–25.0 μg/ml. The method was highly accurate as supported by the fact that there was a high percentage of drug recovered at low as well as high drug concentrations. Further, the relative standard deviation (R.S.D.) values for repeated injection of the same sample and

Table 1
Validation of HPLC technique for the analysis of azole drugs in mice plasma

	Clotrimazole	Econazole
Sensitivity (μg/ml)	0.2	0.2
Linearity		
Range (μg/ml)	0.2–25.0	0.2–25.0
Regression equation	$y = 310871x - 163146$	$y = 284741x - 66944$
Correlation co-efficient (R^2)	0.9976	0.9984
Recovery range (%)	93.4–100.6	91.8–100.5
Accuracy		
Recovery at low concentration (1 μg/ml), %	99.1	99.4
Recovery at high concentration (10 μg/ml), %	99.0	99.6
Precision		
Intra-day R.S.D. (%)		
With sample injected 5 times	0.202	0.214
With sample prepared 5 times	0.927	0.803
Inter-day R.S.D. (%)		
With sample injected 5 times	0.211	0.256
With sample prepared 5 times	0.977	0.892

The regression equation in case of tissue homogenate (taking liver from normal mouse as an example) was $y = 76687x - 45149$ ($R^2 = 0.9972$) for clotrimazole and $y = 471565x - 160436$ ($R^2 = 0.9955$) for econazole. The recovery range was 87.1–95.6% for clotrimazole and 87.5–97.7% for econazole. The sensitivity, linearity, accuracy and precision were similar to that obtained in case of plasma. Therefore, the same analytical technique was employed for estimating drug levels in tissue homogenates.

repeatedly prepared samples, performed on different days or on the same day were <1.5% which confirms that the method was sufficiently precise for each drug. Similar results were obtained when the analytical technique was employed for estimating the drug levels in tissue homogenates (Table 1).

Following the i.v. administration of free clotrimazole/econazole to mice, the plasma drug concentration declined rapidly after the initial (instantaneous) high levels until after 3 h no drug could be detected in plasma (Fig. 1). In case of oral-free clotrimazole/econazole the drugs were detectable up to 3–4 h in plasma. Following

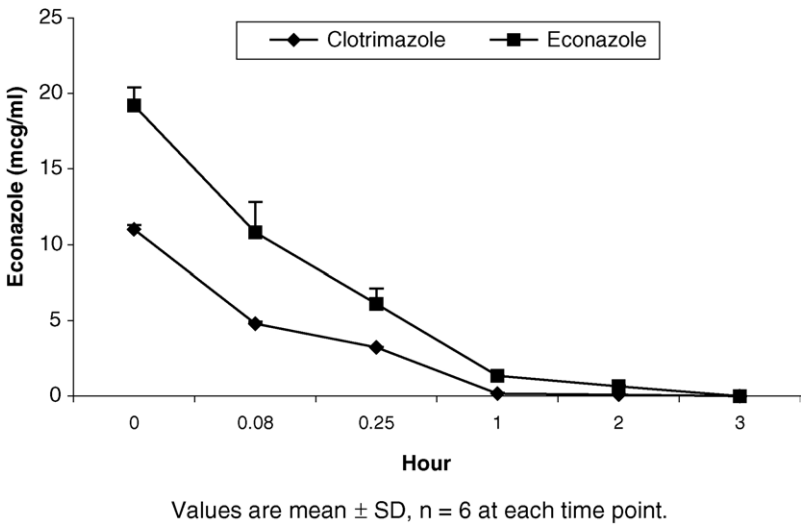

Fig. 1. Plasma drug profile following i.v. administration of free clotrimazole/econazole to mice.

a single oral administration of drug-loaded PLG or alginate nanoparticles, each drug was detectable in the plasma from 3 h to 5 days. In case of alginate nanoparticles, however, econazole was detectable for 6 days. The initial lag phase of 3 h might be attributed to the time taken by the formulation to cross the intestinal barrier and the subsequent release of drugs. The results indicate that it was possible to have a slow and controlled release of azole drugs by encapsulating them into polymeric nanoparticles, a feature not obtained with free drugs alone (Fig. 2). Pharmacokinetic calculations further supported this logic because there was a significant ($p < 0.001$) enhancement in AUC and hence the relative/absolute bioavailability of each drug when administered in the encapsulated state compared with free drugs (Table 2). Specifically, the bioavailability of oral-free azoles which were otherwise poor (0.35 for clotrimazole and 0.37 for econazole) improved several folds. Two other important observations were made from the pharmacokinetic data. Firstly, econazole formulations (PLG or alginate) proved to be better compared with its clotrimazole counterpart because the AUC of encapsulated clotrimazole was significantly lower ($p < 0.001$) compared with the AUC of encapsulated econazole. Secondly, for each azole drug, the enhancement of AUC/bioavailability was significantly more ($p < 0.001$) in case of the alginate formulation compared with the PLG formulation.

The better drug delivery potential of alginate nanoparticles could be explained by the fact that alginate and chitosan are known to possess bioadhesive characteristics which would help in adherence of the

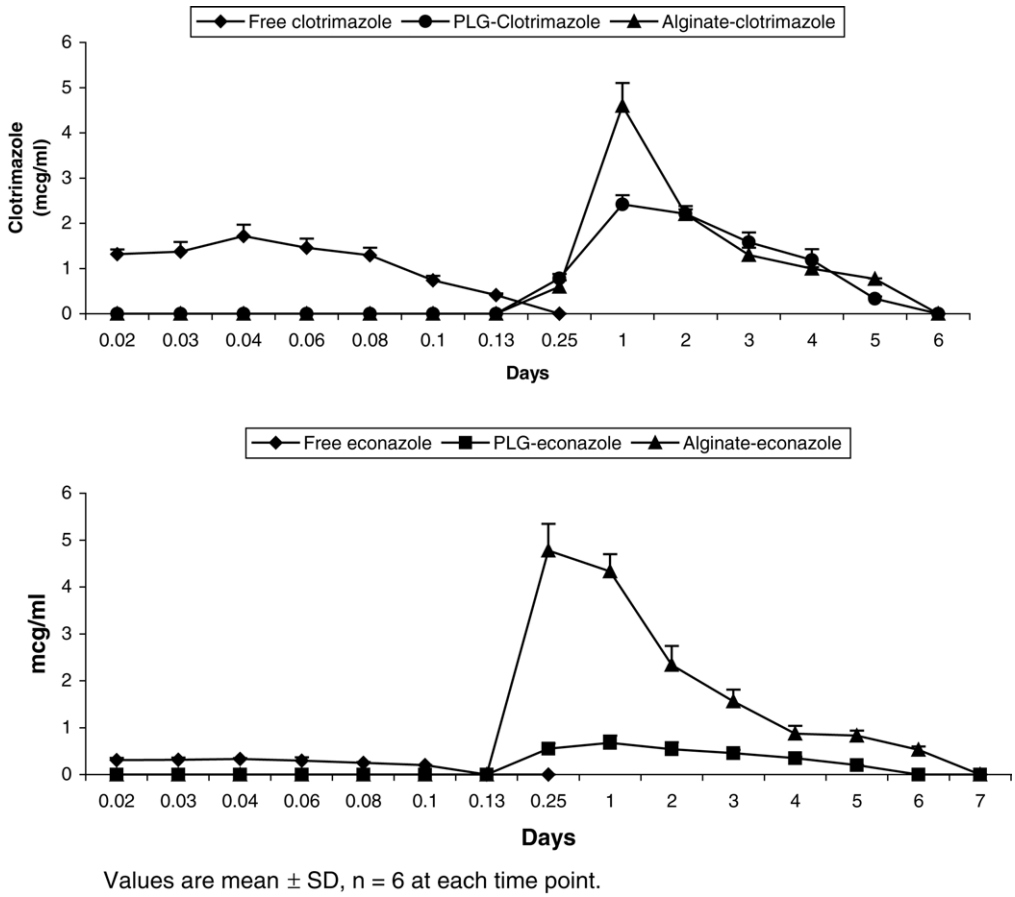

Fig. 2. Plasma drug profile following oral administration of PLG/alginate nanoparticle-encapsulated azole drugs and free drugs to mice.

Table 2
Bioavailability of nanoparticle-encapsulated azole drugs as compared to free drugs in mice

	Pharmacokinetic parameters		
	$AUC_{0\text{-}t}$ (μg h/ml)	Relative bioavailability	Absolute bioavailability
Clotrimazole			
i.v.-free drug	2.61 ± 0.29	–	1
Oral-free drug	0.35 ± 0.03	1	0.13
PLG nanoparticles	14.80 ± 2.04	42.29	5.67
Alginate nanoparticles	46.11 ± 4.94	131.74	17.67
Econazole			
i.v.-free drug	6.27 ± 1.15	–	1
Oral-free drug	0.37 ± 0.06	1	0.06
PLG nanoparticles	58.19 ± 4.00	157.27	9.28
Alginate nanoparticles	190.06 ± 10.00	513.68	30.31

Values for AUC are mean ± S.D. of six mice

Table 3
Tissue drug levels following a single oral administration of azole drug-loaded nanoparticle formulations to mice

	Clotrimazole		Econazole	
	PLG nanoparticles	Alginate nanoparticles	PLG nanoparticles	Alginate nanoparticles
Day 1				
Lungs	1.41 ± 0.18	1.71 ± 0.20	2.13 ± 0.30	2.36 ± 0.30
Liver	2.13 ± 0.22	2.42 ± 0.30	2.78 ± 0.32	2.87 ± 0.31
Spleen	2.13 ± 0.21	2.40 ± 0.21	2.66 ± 0.25	2.93 ± 0.24
Small intestine	1.08 ± 0.17	1.25 ± 0.21	1.32 ± 0.21	1.44 ± 0.26
Kidneys	0.83 ± 0.11	0.78 ± 0.17	1.24 ± 0.21	1.33 ± 0.21
Day 3				
Lungs	0.78 ± 0.14	0.82 ± 0.18	0.82 ± 0.16	0.85 ± 0.14
Liver	1.27 ± 0.18	1.34 ± 0.24	1.08 ± 0.15	1.12 ± 0.11
Spleen	1.17 ± 0.18	1.16 ± 0.19	0.94 ± 0.18	0.88 ± 0.14
Small intestine	Not detectable	Not detectable	Not detectable	Not detectable
Kidneys	Not detectable	Not detectable	Not detectable	Not detectable
Day 7				
Lungs	0.26 ± 0.02	Not detectable	0.26 ± 0.01	0.31 ± 0.01
Liver	0.27 ± 0.02	Not detectable	0.27 ± 0.03	0.61 ± 0.01
Spleen	0.26 ± 0.03	Not detectable	0.25 ± 0.03	0.29 ± 0.07
Small intestine	Not detectable	Not detectable	Not detectable	Not detectable
Kidneys	Not detectable	Not detectable	Not detectable	Not detectable
Day 8				
Lungs	Not detectable	Not detectable	Not detectable	<LOQ[a]
Liver	Not detectable	Not detectable	Not detectable	<LOQ[a]
Spleen	Not detectable	Not detectable	Not detectable	<LOQ[a]
Small intestine	Not detectable	Not detectable	Not detectable	Not detectable
Kidneys	Not detectable	Not detectable	Not detectable	Not detectable

Values are mean ± S.D., $n = 5$ at each time point. In case of oral-free clotrimazole, drug levels at 12 h were: lungs, 1.71 ± .27 μg/ml; liver, 2.09 ± 0.31 μg/ml; and spleen, 2.03 ± 0.32 μg/ml homogenate. In case of oral-free econazole, drug levels at 12 h were: lungs, 1.71 ± 0.27 μg/ml; liver, 2.09 ± 0.31 μg/ml; and spleen, 2.03 ± 0.32 μg/ml homogenate.

[a] Drugs were detectable but less than the limit of quantification.

formulation to the intestinal mucosa for prolonged time periods thereby increasing the time period available for its absorption (Tonnesen and Karlsen, 2002; Pandey and Khuller, 2004c). Chitosan itself is also known to modulate the epithelial gap junctions and aid in the transport of macromolecules (Hejazi and Amiji, 2003). For both the formulations, however, it is likely that the nanoparticles apart from being in the circulation were distributed to various tissues from where the drugs were slowly released as the polymer(s) underwent biodegradation (this aspect is being evaluated further). Therefore, the encapsulated drugs should be detectable for a longer duration in the tissues in contrast to the free drugs at equivalent doses. Indeed, we observed that the drugs were present in all the tissues on day 1 (Table 3). Subsequently, the drugs were undetectable in the small intestine and kidneys on day 3, but continued to be present in lungs, liver and spleen. It seems that the nanoparticles were initially localized in the intestine and later on in the other tissues (except the kidneys), thus explaining the sustained release of drugs. Both the azole drugs were detectable in the tissues till day 7 in case of PLG nanoparticles while in the mice dosed with alginate nanoparticles, clotrimazole and econazole were detectable till day 6 and 8, respectively. Oral-free drugs, on the other hand, were not detectable in the tissues beyond 12 h of administration (Table 3).

So far, attempts to enhance the oral bioavailability of azoles have focused on the use of cyclodextrins, which had limited success (Hostetler et al., 1992). Other workers have reported on the encapsulation of itraconazole in PLG nanoparticles, however, the formulation was not evaluated further (Prakobvaitayakit and Nimmannit, 2003). Our results clearly show that nanotechnology is a powerful tool, which can be employed to improve the oral bioavailability of drugs. Although the present communication is restricted to the encapsulation of azole drugs, similar observations were reported for other antibiotics from our laboratory (Pandey et al., 2003; Sharma et al., 2004). Out of the two formulations evaluated, alginate nanoparticles certainly have an edge over PLG nanoparticles in terms of economy, simplicity of preparation, duration of sustained drug release and drug bioavailability. One or both of the formulations need to be assessed further for their therapeutic potential and might serve to simplify oral antifungal chemotherapy.

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