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Original Article

Pharmacokinetic Exploration of Sublingual Route for the Enhanced Bioavailability of Tacrolimus with Rabbit as Animal Model

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ABSTRACT

Introduction: The study aimed to assess the bioavailability of two sublingual formulations i.e., fast-dissolving films and tablets of the poorly bioavailable immunosuppressant drug, tacrolimus, and to compare with its marketed oral capsule formulation. **Materials and Methods:** Optimized film and tablet formulations of tacrolimus for sublingual administration were developed. Stability studies were performed at room temperature as well as at 40±2°C/75±2% relative humidity for 6 months. The *in vivo* pharmacokinetic studies were performed with rabbits as the animal model. Different pharmacokinetic parameters like $t_{max,}$ C_{max}, AUC, $t_{1/2}$ and Ke were established and they were analyzed statistically by the student t-test. **Results**: The stability analysis showed a satisfactory stability profile of all the dosage forms in a six-month study. The *in vivo* study of the developed formulations showed better pharmacokinetic performance when compared with the marketed oral formulation. There was approximately a 2 and 1.8-fold increase in the relative bioavailability of the film and the tablet formulations respectively. **Conclusion:** The development of tacrolimus into sublingual dosage forms could improve the *in vivo* performance of the drug candidate.

Keywords: Tacrolimus, Sublingual, Bioavailability, Rabbit.

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INTRODUCTION

A big challenge for clinicians in organ transplantation is the rejection of allograft and the patients are often required to comply with the lifelong use of immunosuppressants. Since the 21st century, tacrolimus has been proven to be a boon in the area of organ transplantation and has presented superior results when compared to other immunosuppressant drugs in terms of effective tolerance, reduced side effects, decreased risk of organ rejection and improved organ survival in post-transplant patients.[1] It was first approved in USA to prevent organ rejection in patients receiving allogeneic liver in 1994, kidney in 1997, and heart in 2006.[2]

Tacrolimus binds to a cytosolic protein known as FKBP 12 which then specifically and competitively inhibits calcineurin, resulting in the calcium dependent inhibition of T-helper cell dependent B-cell proliferation, T-cell activation and, thus the formation of lymphokines including interleukins, γ-interferons etc. The suppression of cytotoxic lymphocytes is the main cause of graft rejection.

Tacrolimus is a class II Biopharmaceutics Classification System drug. Tacrolimus exhibits very low aqueous solubility and extensive presystemic metabolism resulting in poor oral bioavailability (approximately 21%). Its pharmacokinetic profile exhibits large inter/intrasubject variability. The half-life of tacrolimus is 8.7 to 11.3 hr and the mean t_{max} was reported to be as 1.5 to 2.0 hr. The drug is a known substrate for the enzymes-P450 3A4(CYP3A4) and P-gp.[3]

The low and erratic oral bioavailability of tacrolimus and the incidence of nausea, vomiting, and gastroparesis in post-transplant patients necessitate alternative routes of administration especially in the early postoperative period to improve its therapeutic efficacy. Oral mucosal administration may be used for patients who face difficulty to tolerate oral drug therapy in both inpatient and outpatient settings. Sublingual delivery allows for direct entry of the drug into the systemic circulation, thus bypassing barriers such as the aqueous milieu of the

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gastrointestinal tract, P-gp and P450 3A4 (CYP3A4), and the first-pass metabolism in the liver.[4]

To our knowledge, studies on the pharmacokinetic parameters after the administration of sublingual dosage forms of tacrolimus have not been reported so far. The present investigation reports the improved pharmacokinetics of tacrolimus from sublingual fast-dissolving film and tablet formulations compared to the marketed formulation with rabbit as the animal model.

MATERIALS AND METHODS

Tacrolimus monohydrate was procured from Yarrowchem (Mumbai, India). Lox spray was purchased from Neon laboratories, and Tincture benzoin from Wallis Pharmaceutical. Carboxy methyl cellulose, was procured from Himedia Lab Pvt. Ltd., (Mumbai, India). Acetonitrile and water (HPLC grade) were purchased from Sigma-Aldrich Chemicals (Bangalore, India).

Development of tacrolimus loaded fast dissolving films and tablets

The preparation and evaluation of tacrolimus-loaded fast-dissolving films and tablets had already been discussed in earlier publications by Mohanan J. and co-authors.[5,6] To brief, to enhance the solubility of the drug, firstly, inclusion complexes with β-cyclodextrin were developed and characterized followed by their formulation into fast-dissolving films and tablets for sublingual administration. The optimized formulations were further selected for their *in vivo* bioavailability study in rabbits. The composition and method of preparation of the optimized formulations are given in Table 1.

Stability studies

The optimized film and tablet formulations in closed vials were placed in a humidified chamber at room conditions and at 40±2°C and 75±2% relative humidity for 6 months.

The film formulations were evaluated for appearance (in terms of transparency, homogeneity, and flexibility), uniformity of drug content, *in vitro* disintegration time and *in vitro* drug dissolution studies. The tablet formulations were evaluated for uniformity of drug content, friability, weight variation, *in vitro* disintegration time, and *in vitro drug* dissolution studies.

Pharmacokinetic studies

The *in vivo* pharmacokinetic study was conducted at Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, Tamil Nadu, India after obtaining approval from the Institutional Animal Ethics Committee (no. IAEC/68/SRIHER/802/2022). The experiments were performed following their protocol.

Experimental conditions

The environmental temperature was maintained in the range of 19°C-23°C and the relative humidity at 50%-70%. 12-15 air changes/hr were maintained in animal confinements. The animals were exposed to artificial light for a period of 12 hr and to darkness for 12 hr. Rabbits were housed in stainless steel cages individually and they were provided with a laboratory feed diet and water *ad libitum*.

An equivalent drug dose of 1.5mg/kg body weight was administered sublingually in the form of film and tablet. Orally, the same dose of the marketed formulation (Prograf Capsules) was given as a suspension in 0.3% carboxy methyl cellulose (Figure 1).

Table 1: The composition and method of preparation of the optimized formulations.

Formulation	Composition	Method of preparation
Film	-Tacrolimus-β-cyclodextrin inclusion complex (1:2 molar ratio) equivalent to 1 mg drug -HPMC E5 (3.0% w/v) -Croscarmellose sodium (11.7% w/w) -PEG 400 (2% v/v) -Menthol (1% w/v) -Saccharine sodium (0.8% w/v)	Solvent casting method Solvents used: Distilled water and methanol (3:7)
Tablet	-Tacrolimus-β-cyclodextrin inclusion complex (1:2 molar ratio) equivalent to 1 mg drug - Croscarmellose sodium (12 mg) -Magnesium stearate (1.5 mg) -Talc(1 mg) -Mannitol (12 mg) -Citric acid (1.25 mg) -Microcrystalline cellulose q.s.	Direct compression

Experiment procedure

Three rabbits of New Zealand white species were used for the study in three phases. Following a period of acclimatization for six days, the animals were administered with the marketed oral formulation (Phase 1). After appropriate washout periods, the animals were administered the sublingual film of tacrolimus (Phase 2) and the sublingual tablets (phase 3). Approximately 1.25 mL of blood sample volumes were collected through the marginal ear vein as per standard protocol (anaesthetized using a local anaesthetic agent) before dosing (0 hr) and at 15 min, 30 min, 1, 1.5, 2, 4, 12, and 24 hr after the administration of each treatment in heparinized tubes. The collected blood samples were centrifuged for 10 min, at 3500 rpm and the plasma separated was kept at temperature appropriately of -80°C until further analysis.

Estimation of tacrolimus in plasma

The HPLC system

The plasma drug concentrations were measured by the official (USP) HPLC-UV method with slight modifications.[7] The equipment used was Agilent 1220 infinity gradient LC system.

Preparation of calibration curve of tacrolimus in rabbit plasma

The standard drug was spiked into rabbit plasma and diluted to obtain concentrations of 100, 200, 300, 400, 500, 600 and 700 ng/µL. All the solutions were vortexed for 15 sec and centrifuged at 5000 rpm for 15 min. Further, the upper layer was filtered through a 0.2 µm millipore filter and samples were injected into the liquid chromatographic column.

Pharmacokinetic analysis

A mean plasma drug concentration-time profile was constructed. The peak plasma tacrolimus concentration, $C_{max,}$ the time to reach this C_{max} (t_{max}), elimination half-life ($t_{1/2}$), elimination rate constant (Ke) and the area under the plasma drug concentration-time curve from zero time to 24 hr (AUC_{0-24}) were determined employing non compartmental analysis. Also the relative bioavailability of test products was determined with respect to the reference product.

Statistical analysis

The various pharmacokinetic parameters calculated were subjected to a one-way analysis of variance and student t-test in Microsoft excel.

RESULTS

Stability studies

Both the optimized formulations stored at room temperature as well as at 40±2°C/75±2% RH showed satisfactory results and were found to be stable (Tables 2 and 3). The *in vitro* disintegration time of films and tablets was slightly decreased. Their drug content was found to be slightly reduced but still, they were in the conventional acceptable range of 90-110% of the label claim for end-of-shelf-life specifications. Similar results were reported previously, which may probably be attributed to thermal degradation and/or hydrolysis.[8,9]

Pharmacokinetic studies

Observation of the animals

All the animals during the experiment were found to be normal. No test formulation item-related mortality or morbidity was detected in any of the test animals and they did not exhibit any signs of toxicity. There was no decrease in body weight observed throughout the experimental period.

HPLC Analysis-Calibration curve

The calibration curve obtained from the HPLC analysis of the plasma spiked standard drug solutions is presented in Figure 2. The analysis of linear regression of the calibration plot in plasma showed a good linear relationship for the concentration ranges of 100-700 ng/µL of tacrolimus. The regression equation was y= 1.949x -28 with a correlation coefficient (R^2) of 0.998.

Plasma analysis

Figure 3 shows the plasma concentration time profiles after the oral administration of a single dose (1.5 mg tacrolimus/kg body weight) of marketed formulation and sublingual administration of the developed film and tablet to rabbits. At each sampling time point, the plasma tacrolimus levels after sublingual administration were significantly higher than those after oral administration.

All the pharmacokinetic parameters which were derived from the plasma concentration profile are given in Table 4. An obvious increase in the plasma levels of tacrolimus from sublingual films and tablets was there when compared to those of the marketed formulation. The C_{max} obtained with sublingual films and

Figure 1: Oral and sublingual administration of test items to rabbits.

Table 2: Stability studies of the optimized film (mean±SD, *n*=3).

Evaluation parameters	Storage conditions	Zero time	6 months
Appearance	RT	+++	++
	40±2°C/75±2%		+
In vitro disintegration time (s)	RT	27.28±3.16	24.51±6.7
	40±2°C/75±2%		22.73±7.37
Uniformity of drug content (%)	RT	98.42±0.94	97.14±1.28
	40±2°C/75±2%		93.47±2.96
In vitro drug release (%)	RT	83.1±3.17	81.97±4.10
	40±2°C/75±2%		79.96±5.24

Table 3: Stability studies of the optimized tablet (mean±SD, *n*=3).

Evaluation parameters	Storage conditions	Zero time	6 months
Weight variation (mg)	RT	101.95±4.40	103.54±3.30
	40±2°C/75±2%		101.37±2.12
Friability (%)	RT	0.749	0.861
	40±2°C/75±2%		0.970
In vitro disintegration time(s)	RT	34.33±5.32	32.86±4.8
	40±2°C/75±2%		28.98±6.13
Uniformity of drug content (%)	RT	96.48±2.47	94.30±2.06
	40±2°C/75±2%		92.23±3.54
In vitro drug release (%)	RT	97.87±1.49	96.10±2.32
	40±2°C/75±2%		94.47±4.19

Table 4: Comparison of pharmacokinetic parameters of various formulations (mean±SEM, *n*=3).

Parameters	Marketed formulation	Film	Tablet
C_{max} (ng/mL)	148.95±10.09	290.65±8.23	266.27±9.18
T_{max} (h)	1	0.5	0.5
AUC_{0-24} (ng.hr/mL)	696.76±32.13	1445.57±28.68	1311.62±67.21
$AUC_{0-\infty}$ (ng.hr/mL)	803.9±37.72	1561.99±76.35	1444.77±83.69
Ke (h^{-1})	0.06±0.011	0.07±0.014	0.05±0.001
$t_{1/2}$ (h)	11.45±1.69	11.67±3.04	12.82±0.31

tablets were 290.6467±8.23 ng/mL and 266.2667±9.18 ng/mL respectively which were found to be comparatively higher than that obtained with the marketed formulation (148.95±10.09). T_{max} observed for the marketed formulation was 1 hr whereas it was found to be 0.5 hr for both the sublingual formulations. Comparable $t_{1/2}$ values were obtained for all the formulations. There was a notable difference in the AUC_{0-24} between test formulations and the reference product; 1445.57±28.68 ng. hr/mL and 1311.615±67.21 ng. hr/mL respectively for sublingual film and tablet and 696.76±32.13 ng. hr/mL for marketed formulation. Moreover, there was approximately a 2 and 1.8 -fold increase in bioavailability observed for film and tablet respectively compared to reference marketed formulation.

DISCUSSION

The optimum outcome for an immunosuppressant like tacrolimus is when its physicochemical and pharmacokinetic limitations are successfully overcome. The extensive presystemic metabolism in the intestine and liver has greatly influenced the *in vivo* potential of tacrolimus which has led to its inadequate therapeutic efficacy. Therefore, the issue of the drug's poor and unpredictable oral bioavailability would ideally be resolved by obtaining optimal concentration in blood with a minimal dose.

Currently reported target therapeutic concentrations of tacrolimus in different transplant types usually range from 5-20 ng/mL, though it may vary for specific organs transplanted[10]

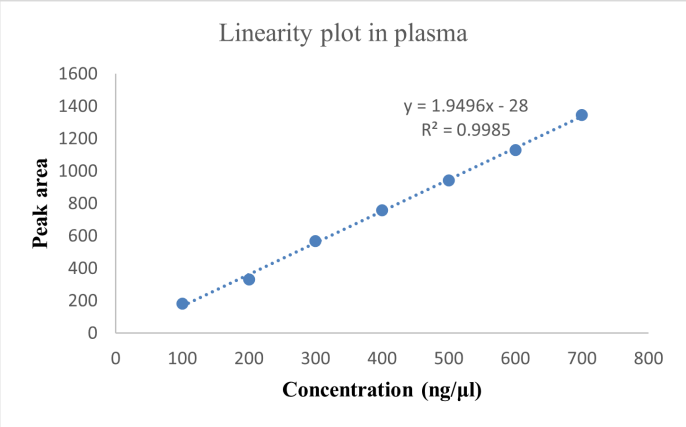

Figure 2: Calibration curve of tacrolimus in plasma.

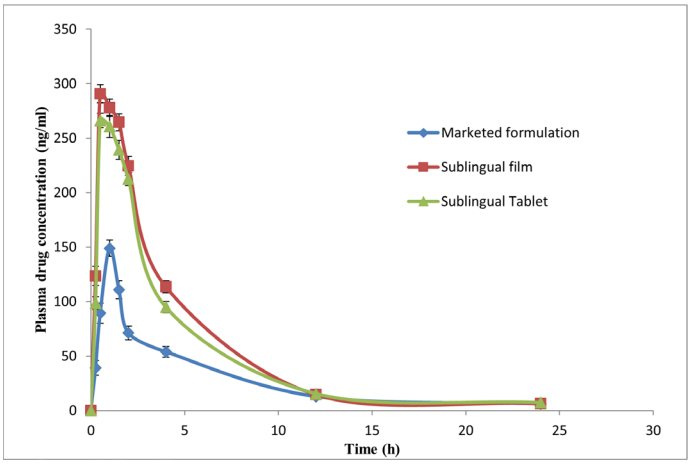

Figure 3: Plasma concentration-time graph of various formulations (mean±SEM, *n*=3 for each formulation).

and for the first 2-3 months following organ transplantation, the target trough concentrations in the blood are 8 to 10 ng/mL.[11] A decreased trough level of tacrolimus during the initial post-transplant periods has been associated with a greater rate of acute rejection. Moreover, lower allograft survival has been linked to increased intra-individual variability in tacrolimus trough concentration.[12]

Presently tacrolimus is available as solutions and solid dispersion-based capsules for oral administration and as IV infusions. Commercial formulations for oral use are reported to have only 25% bioavailability.[13] Intravenous tacrolimus administration requires a continuous infusion and is associated with nephro as well as some neurotoxicities.[14]

There are many studies reported for the enhancement of oral bioavailability of tacrolimus. A pharmacokinetic study performed in rats by Lee *et al.* (2016) revealed that a supersaturable formulation was effective in boosting its dissolution and oral absorption.[15] Nekkanti *et al.* (2016) conducted an *in vivo* study in rats with a proliposome formulation of tacrolimus and showed a considerable improvement in the rate and absorption.[13]

Research efforts have also been made by exploiting alternate routes for its administration. Lee *et al.* (2019) determined various pharmacokinetic parameters of tacrolimus following intramuscular injection in cynomolgus monkeys. The study suggested intramuscular injection as an alternative dosing route.[12] Sakai *et al.* (2004) successfully explored the rectal route and met with improved pharmacokinetics of tacrolimus.[16]

Several human clinical studies have been reported on the utility of sublingual tacrolimus administration in the prevention of allograft rejection.[17-19] To our knowledge, no previous studies on the pharmacokinetics of any tacrolimus formulation in animal models have been reported.

The present study has explored the sublingual pharmacokinetics of tacrolimus employing rabbits as the animal model. Considerably high drug concentration in plasma was attained (Figure 3) with sublingual administration compared with oral, as can be seen from the significantly higher AUC_{0-24}, 1445.57±28.68 ng.hr/mL for the film (p<0.05) and 1311.615±67.21 ng.hr/mL for the tablet (p<0.05) in comparison with for marketed formulation (696.76±32.13 ng.hr/mL). C_{max} was also significantly increased (290.65±8.23 ng/mL, p< 0.05 for film and 266.2667±9.18 ng/mL, p<0.05 for tablet) compared to the marketed formulation (148.95±10.09 ng/mL) which may be due to quick and better drug absorption through sublingual mucosa. There was a shift for t_{max} from 1 hr to 0.5 hr with oral and sublingual routes respectively, but these differences were not statistically significant (p>0.05). The notable hike observed for relative bioavailability (~2 and 1.8 fold increase for film and tablet than that of reference marketed formulation) indicates that more amount of the drug got entry into the blood circulation and the reason may be attributed to the inherent advantages of the sublingual route including bypassing of presystemic metabolism, high permeability, etc.

Moreover, the pharmacokinetic parameters obtained from sublingual film were also compared with the paramerters of tablets. Even though AUC_{0-24} and t_{max} of the film were higher than that of the tablet, the difference was concluded to be statistically insignificant (p>0.05). But the differences observed in the case of C_{max} between them were found to be statistically significant (p<0.05).

CONCLUSION

Successful development of dosage forms of drugs possessing poor physicochemical and pharmacokinetic properties has been always a big challenge for researchers. The current research work demonstrated the utility of the sublingual route for the improved therapeutic outcome of a poorly bioavailable drug candidate like tacrolimus. The *in vivo* performance of previously developed fast-dissolving films and tablets was compared to marketed oral formulation in rabbits. The sublingual administration resulted in an improved pharmacokinetic profile than the oral route with higher relative bioavailability. So, sublingual tacrolimus can be

considered as an alternative to oral administration which may lead to better patient compliance, reduced medication cost, and improved bioavailability.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

C_{max}: Peak plasma concentration; **t_{max}:** Time to reach; **t1/2:** elimination half-life; **Ke:** elimination rate constant; **AUC:** Area Under the plasma drug concentration-time Curve; **RT:** Room Temperature; **HPMC:** Hydroxy Propyl Methyl Cellulose; **PEG:** Poly Ethylene Glycol; **HPLC:** High Performance Liquid Chromatography; **RPM:** Revolutions per minute.

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