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# Development and Validation of HPTLC Method for Estimation of Tacrolimus in Formulations

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**A new, simple, and rapid high-performance thin-layer chromatographic method with a derivatization procedure was developed and validated for quantitative determination of tacrolimus. Tacrolimus was chromatographed on silica gel 60 $F_{254}$ TLC plate using toluene–acetonitrile–glacial acetic acid (6:4:0.1, by volume) as mobile phase. Tacrolimus was visualized using a derivatization reagent containing anisaldehyde–sulfuric acid in absolute alcohol and quantified by densitometric analysis in the reflectance mode at 675 nm. The method was found to give compact spots for the drug ($R_f$ = 0.40 ± 0.03). The linear regression analysis data for the calibration plots showed good linear relationship with $r^2$ = .9989 in the concentration range 100–800 ng/spot. The method was validated for precision, recovery, repeatability, and robustness as per the International Conference on Harmonization guidelines. The minimum detectable amount was found to be 28.90 ng, whereas the limit of quantitation was found to be 97.04 ng. Statistical analysis of the data showed that the method is precise, accurate, reproducible, and selective for the analysis of tacrolimus. The method was successfully employed for the estimation of equilibrium solubility and quantification of tacrolimus as a bulk drug and in commercially available capsules and in-house developed self-microemulsifying formulations.**

**Keywords** tacrolimus; HPTLC; validation; solubility; self-microemulsifying formulations

## INTRODUCTION

Tacrolimus, a macrolide antibiotic (Figure 1) isolated from *Streptomyces tsukubaensis*, is a potent immunosuppressant used clinically in the prevention of organ rejection after liver, kidney heart, lung, pancreas, and bone marrow transplantation. Today, this calcineurin inhibitor forms the basis of most of the immunosuppressive protocols after organ transplantation as a primary and rescue agent (Christine & Susan, 2004; Shapiro, 1999; Spencer, Goa, & Gillis, 1997). Because of its potent nature and safety advantages over cyclosporine A, several researchers have focused on improving its delivery by solid dispersions (Arima, Yunomae, Miyake, & Uekama, 2001; Yamashita et al., 2003) and liposomes (Dutta, Mezei, & Lee, 1998; McAlister, Keshavamurthy, & Lee, 1999). Various methods such as enzyme-linked immunosorbent assay (Beysens, Wijnen, & Beuman, 1991; Tamura, Kobayashi, & Rnhimoto, 1987), high-performance liquid chromatography (HPLC)–mass spectrometry (Alak, Moy, & Menard, 1997; Deters, Kaever, & Kirchner, 2003), and chemiluminescence (Takada, Hashi, Yoshikawa, & Muranishi, 1990) are reported in literature for analysis of tacrolimus from biological fluids. These methods are very sensitive and useful for assaying tacrolimus for pharmacokinetic studies and therapeutic drug monitoring. Calibration curves of these methods range in ng/mL scale and are not appropriate for determining tacrolimus in pharmaceutical products without considerable prior dilution. Furthermore, most of these methods are highly sophisticated, costly, and time-consuming and require special sample preparation. Another problem in the analysis of tacrolimus is its low absorptivity at conventionally used detection wavelengths. Tacrolimus exhibits a maximum absorbance at ~205 nm, which poses difficulties in its estimation using ultraviolet spectroscopy (Kino et al., 1987; Wallemacq & Reding, 1993). Therefore, reported reverse-phase (RP)-HPLC-based methods require working at lower wavelengths such as at 215 nm (Akashi, Nefuji, Yoshida, & Hosoda, 1996; Namiki, Fujiwara, Kihara, Koda, & Hane, 1995). Further, RP-HPLC-based separation methods may not be suitable for the determination of drug from lipid-based delivery systems such as self-microemulsifying

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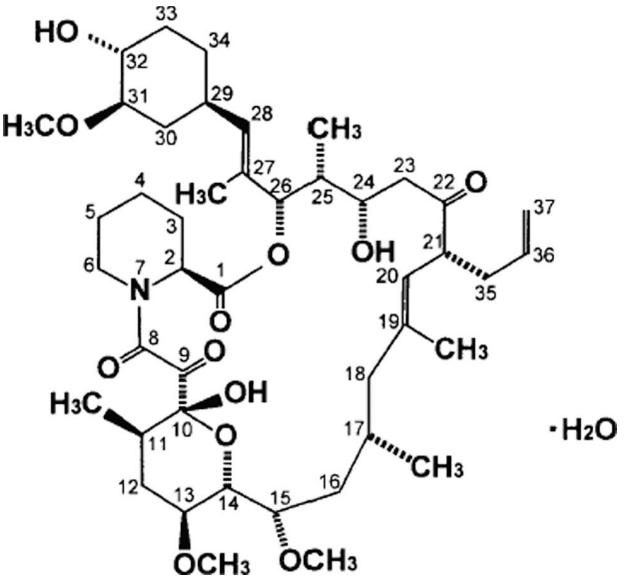


FIGURE 1. Chemical structure of tacrolimus.

formulations. These formulations contain various lipophilic excipients that are not soluble in commonly used organic solvents used in RP-HPLC methods. Further, extraction of drug from such lipophilic excipients may not be achieved easily, and such excipients may get adsorbed on stationary phase. Hence, analysis of tacrolimus, particularly from lipid-based delivery systems, would be difficult with respect to identification of suitable solvents and stationary phase.

In view of this, high-performance thin layer chromatography (HPTLC)-based methods could be considered as a good alternative as they are being explored as an important tool in routine drug analysis. Major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with same or different parameters. Furthermore, in case of HPTLC, there are no restrictions on the choice of solvents and mobile phases; drug and lipophilic excipients can be dissolved in a suitable solvent that would evaporate during spotting on TLC plate leaving behind analyte as a thin band. Therefore, for such methods, extraction procedure is not required always and could be developed for analyzing drug without any interference from excipients. Further, to our knowledge, HPTLC method of analysis has not been explored for tacrolimus till date. In view of this, the present study describes the development of simple, rapid, economic, and validated HPTLC method for routine estimation of tacrolimus from bulk and pharmaceutical dosage forms such as marketed capsules (Pangraf™) and self-microemulsifying formulations developed in-house.

## MATERIALS AND METHODS

### Materials

Tacrolimus was obtained as gift sample from Glenmark Pharmaceuticals Ltd., Nasik, India. Capsules of tacrolimus (Pangraf™ 5 mg, Panacea Biotec Ltd., Delhi, India) were purchased. Methanol, acetonitrile, toluene, and sulfuric acid (98%) were obtained from Qualigens Fine Chemicals, Mumbai, India. Absolute alcohol, *p*-anisaldehyde, and ethyl oleate were obtained from S.D. Fine Chem. Ltd., Mumbai, India. Glycofurol was purchased from E. Merck, Mumbai, India. Cremophor-EL, Solutol HS-15 (BASF, Mumbai, India), Capmul MCM C8/10 (Abitec Corporation, Janesville, USA), and Transcutol (Colorcon Asia Pvt. Ltd., Mumbai, India) were obtained as gift samples. All chemicals and reagents were of analytical grade and used as received.

### Instrumentation

The HPTLC system (Camag, Muttenz, Switzerland) consisted of Limomat V semi-automatic sample applicator with 100-μL syringe connected to a nitrogen cylinder, a twin trough chamber (10 × 10 cm), a derivatization chamber, and a plate heater. Pre-coated silica gel 60 $F_{254}$ TLC plates (20 × 20 cm, layer thickness 0.2 mm, Code No. 1.05554.0007, Lot No. 0B312022E, Merck, Darmstadt, Germany) were used as stationary phase. TLC plates were pre-washed twice with 10 mL of methanol and activated at 80°C for 5 min prior to sample application. Densitometric analysis was carried out using a TLC scanner 3 with winCATS software V1.1.3.0, and integration was performed using CAMAG TLC scanner/Integrator system LCI-100.

### HPTLC Method and Chromatographic Conditions

*Sample Application*

The standard and formulation samples of tacrolimus were spotted on pre-coated TLC plates in the form of narrow bands of lengths 6 mm, with 10 mm from the bottom and left margin and with 9-mm distance between two bands. Samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nL/s.

*Mobile Phase and Migration*

Plates were developed using mobile phase consisting of toluene–acetonitrile–glacial acetic acid (6:4:0.1, by volume). Linear ascending development was carried out in 10 cm × 10 cm twin trough glass chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20 min at 25 ± 2°C. Ten milliliters of the mobile phase (5 mL in trough containing the plate and 5 mL in other trough) was used for each development and allowed to migrate a distance of 60 mm, which required 10 min. After development, the TLC plates were dried completely.

*Derivatization Procedure*

The derivatization reagent was prepared by adding 1 mL concentrated sulfuric acid to a solution of 1 mL anisaldehyde in 9 mL absolute alcohol. The reagent was freshly prepared before use. After chromatographic development, the TLC plate was dried with a hair dryer. Derivatization was performed by soaking the developed plate in freshly prepared derivatization reagent for 10 s. After drying, TLC plate was heated for 1 min at 110°C.

*Densitometric Analysis and Quantitation Procedure*

Densitometric scanning was performed on Camag TLC scanner III in reflectance mode and operated by winCATS planar chromatography version 1.1.3.0. The source of radiation utilized was halogen–tungsten lamp. The spots were analyzed at a wavelength of 675 nm.

The slit dimensions used in the analysis were length and width of 5 mm and 0.45 mm, respectively, with a scanning rate of 20 mm/s. These are selected as recommended by the CAMAG TLC Scanner III manual. It covers 70–90% of the application band length, which in the present case is 6 mm. The monochromator bandwidth was set at 20 nm. Concentrations of compound chromatographed were determined from the intensity of diffusely reflected light and evaluated as peak areas against concentrations using linear regression equation.

*Method Validation*

Validation of the developed HPTLC method was carried out as per the International Conference on Harmonization (ICH) guidelines Q2 (R1) for specificity, sensitivity, accuracy, precision, repeatability, and robustness.

*Specificity*

The specificity of the developed method was established analyzing the sample solutions containing tacrolimus from self-microemulsifying formulations and marketed capsules in relation to interferences from formulation ingredients. The spot for tacrolimus in the sample was confirmed by comparing retention factor ($R_f$) values of the spot with that of the standard.

*Sensitivity*

Sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantification (LOQ). Noise was determined by scanning blank spot (methanol) six times. Series of concentrations of drug solutions (20–800 ng/spot) were applied on plate and analyzed to determine LOD and LOQ. LOD was calculated as 3 times the noise level, and LOQ was calculated as 10 times the noise level. LOD and LOQ were experimentally verified by diluting the known concentrations of tacrolimus until the average responses were approximately 3–10 times the *SD* of the responses for six replicate determinations.

*Linearity and Calibration Curves*

Linearity of the method was evaluated by constructing calibration curves at five concentration levels. Calibration solutions of tacrolimus in methanol were prepared with concentrations of 10, 20, 40, 60, and 80 µg/mL by individual weighing. Ten microliters of each solution was applied on the TLC plate to obtain final concentration range of 100–800 ng/spot. The calibration curve was constructed by plotting peak areas obtained from analysis of standard drug solutions versus corresponding concentrations and treated by linear least-square regression analysis.

*Accuracy*

Accuracy of the method was evaluated by carrying the recovery study at three levels. Recovery experiments were performed by adding three different amounts of standard drug, i.e., 80, 100, and 120% of the drug, to the preanalyzed self-microemulsifying formulations and conventional capsules, and the resultant was reanalyzed six times.

*Precision*

Precision was evaluated in terms of intra-day and inter-day precisions. Intra-day precision was determined by analyzing sample solutions of tacrolimus from self-microemulsifying formulation at three levels covering low, medium, and higher concentrations of calibration curve for five times on the same day. Inter-day precision was determined by analyzing sample solutions of tacrolimus at three levels covering low, medium, and higher concentrations over a period of seven days ($n = 5$). The peak areas obtained were used to calculate mean and %*RSD* (relative *SD*) values.

*Repeatability (System Precision)*

Repeatability of measurement of peak area was determined by analyzing different amount of tacrolimus samples covering low, medium, and higher ranges of the calibration curve seven times without changing the position of plate. Repeatability of sample application was assessed by spotting tacrolimus samples covering similar range of calibration curve seven times and analyzing them once.

*Robustness*

By introducing small changes in mobile phase composition, its volume, chamber saturation time, and slight change in the solvent migration distance, the effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 400 ng/spot and the mean and %*RSD* of peak area was calculated.

## Application of Developed Method

*Determination of Equilibrium Solubility*

Solubility of tacrolimus in various excipients was determined by shake flask method. An excess of tacrolimus was added to 1 g of each of the excipient and vortexed to facilitate

the mixing. Mixtures were shaken for 48 h in a reciprocating water bath shaker maintained at room temperature. After 48 h, each tube was centrifuged at 600 × *g* for 10 min, and the insoluble drug was discarded by filtration using a 0.45-μm membrane filter. The filtrate was suitably diluted with methanol and concentration of drug was quantified by a developed HPTLC method.

*Determination of pH-Solubility Profile*

Solubility of tacrolimus in various buffers was determined by shake flask method as described above. Various buffers of pH ranging from 1.2 to 10.6, namely, buffer pH 1.2 (hydrochloric acid–potassium chloride buffer), buffer pH 3.0 (glycine–hydrochloric acid buffer), buffer pH 4.5 (acetate buffer), buffer pH 6.8 (citrate–phosphate buffer), buffer pH 7.4 (phosphate buffer), buffer pH 9 (glycine–sodium hydroxide buffer), and buffer pH 10.6 (carbonate–bicarbonate buffer), were used.

*Tacrolimus Formulations*

Two self-microemulsifying formulations of tacrolimus were developed in-house (Borhade, 2006; Borhade, Nair, & Hegde, 2008), one by mixing Cremophor-EL and Transcutol in a weight ratio of 2:1, with 20% (wt/wt) Capmul MCM C8/10 (formulation F1: for oral delivery of tacrolimus), and another by mixing Solutol HS-15 and glycofurol in a weight ratio of 3:2, with 13% (wt/wt) ethyl oleate (formulation F2: for intravenous delivery of tacrolimus). Both formulations on dilution with water yield microemulsion with mean particle size less than 25 nm as determined by photon correlation spectroscopy using Beckman Coulter N5 plus Submicron Particle Sizer (Coulter Corporation, Fullerton, CA, USA). Formulation F1 equivalent to 5 mg of tacrolimus was filled in size "2" hard gelatin capsules, whereas formulation F2 equivalent to 5 mg of tacrolimus was filled in 5 mL type I borosilicate glass vials, and both the formulations were analyzed using proposed method. Commercially available capsules of tacrolimus, Pangraf™, 5 mg were also used in this investigation to verify the suitability of the method for analysis of tacrolimus from conventional dosage forms. Pangraf™ is a solid dispersion of tacrolimus with hydroxypropyl methyl cellulose, filled in hard gelatin capsule along with other excipients such as lactose and magnesium stearate (Arima et al., 2001; Yamashita et al., 2003).

*Analysis of Tacrolimus in Formulations*

The contents of 10 capsules (each containing 5 mg of tacrolimus) were emptied and intimately mixed. Quantity equivalent to 10 mg of drug was weighed accurately and dissolved in 50 mL methanol. The solution was sonicated for 15 min and then filtered through Whatmann filter paper No. 41. The residue was washed thoroughly with methanol. The filtrate and washings were combined and diluted suitably with methanol to obtain a 40 μg/mL concentration of tacrolimus. Self-microemulsifying formulations containing 10 mg equivalent of tacrolimus were dispersed in 50 mL of methanol and were treated in a similar manner as that of capsules to obtain a stock solution of 40 μg/mL. On TLC plates, 10 μL of these solutions were spotted and analyzed for tacrolimus content using proposed method as described earlier. The possibility of interference from other components of the capsule formulation in the analysis was studied. Placebo self-microemulsifying formulations were analyzed similarly to study the potential interference.

*In Vitro Dissolution Profile Tacrolimus Formulation*

Self-microemulsifying formulation of tacrolimus for oral delivery (formulation F1) was evaluated for in vitro release using USP XXIII apparatus I at 37 ± 0.5°C, at 100 rpm in buffer pH 1.2 and 6.8 (250 mL). During study, 2 mL of aliquots were removed at 15, 30, 60, 90, 120, and 180 min and replaced with fresh buffer. Amount of drug released was determined using developed HPTLC method. In vitro release of Pangraf™ capsules and plain tacrolimus was also determined in a similar manner.

## RESULTS AND DISCUSSION

### Development of Optimum Mobile Phase

Because no literature report of HPTLC method of analysis for tacrolimus for routine analysis was available, selection of mobile phase was carried out on the basis of polarity. A solvent system that would give dense and compact spots with appropriate and significantly different $R_f$ value for tacrolimus was desired. Various solvent systems such as methanol–chloroform, methanol–toluene, toluene–ethyl acetate, hexane–ethyl acetate, hexane–acetone, toluene–acetonitrile, and toluene–acetonitrile–glacial acetic acid were evaluated in different proportions. Among these, the solvent system comprising of toluene : acetonitrile (6:4, vol/vol) gave good separation of tacrolimus from its matrix with an $R_f$ value of 0.43; however, spot of analyte was slightly diffused. The problem was eliminated by adding glacial acetic acid to mobile phase. It was also observed that chamber saturation time and solvent migration distance are crucial in chromatographic separation as chamber saturation time of less than 15 min and solvent migration distances greater than 60 mm resulted diffusion of analyte spot. Therefore, toluene–acetonitrile–glacial acetic acid solvent system in 6:4:0.1 (by volume) proportion with chamber saturation time of 15 min at 25°C and solvent migration distance of 60 mm was used as mobile phase. These chromatographic conditions produced a well-defined compact spot of tacrolimus with optimum migration at $R_f$ = 0.40 ± 0.03 (Figure 2). It also gave a good resolution of analyte from excipients used in various self-microemulsifying formulations and marketed capsule formulation.

### Selection of Derivatization Reagent

Various reagents such as iodine vapors, ceric sulfate, sulfuric acid, and Dragendorff reagent were tried for the identification of

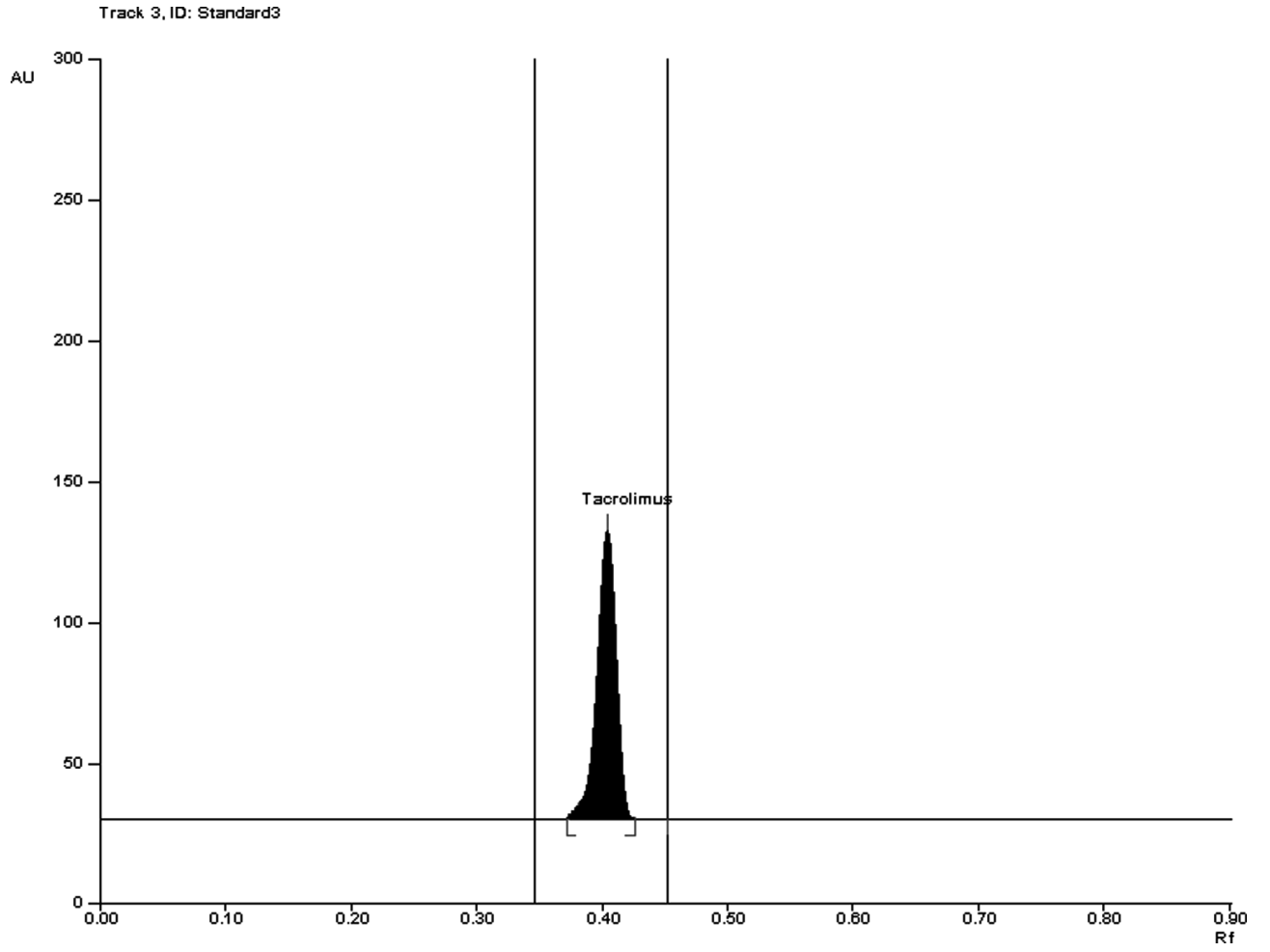


FIGURE 2. Chromatogram of standard tacrolimus (400 ng/spot) using mobile phase toluene–acetonitrile–glacial acetic acid (6:4:0.1, by volume).

tacrolimus but were not suitable for its quantitative determination (Kino et al., 1987). Tacrolimus being a macrolide lactone has structural similarities with macrolide antibiotics, and hence, derivatization reagents such as Folin and Ciocalteu's reagent, molybdophosphoric acid, and reagents based on *p*-anisaldehyde (4-methoxybenzaldehyde) which are used to visualize these antibiotics were studied (Koch, 1998). Except the reagent based on *p*-anisaldehyde, no other reagents could produce color with tacrolimus. Anisaldehyde and sulfuric acid in absolute alcohol (1:1:9, by volume) with activation of plate at 110°C for 60 s produced a strong coloration of tacrolimus yielding well-defined spots that could be used for quantative determination.

## Method Validation

*Sensitivity*

Under the experimental conditions employed, the lowest amount of drug that could be detected was found to be 28.90 ng/spot and the lowest amount of drug that could be quantified was found to be 97.04 ng/spot, with *RSD* <5%.

*Specificity*

Specificity is the ability of an analytical method to assess unequivocally the analyte in the presence of sample matrix. Tacrolimus was separated from excipient with an $R_f$ of $0.40 \pm 0.03$. There was no interfering peak at the $R_f$ value of tacrolimus from excipients such as ethyl oleate, Capmul MCM C8/10, Cremophor-EL, Solutol HS-15, Glycofurol, and Transcutol present in self-microemulsifying formulations. In addition, there was no interference from excipients, hydroxypropyl methylcellulose, and lactose present in commercial capsules, thereby confirming specificity of method.

*Linearity and Calibration Curves*

Linearity of an analytical method is its ability, within a given range, to obtain test results that are directly, or through a mathematical transformation, proportional to concentration of analyte. Method was found to be linear in a concentration range of 100–800 ng/spot ($n$ = 5), with respect to peak area. Figure 3 displays three-dimensional overlay of HPTLC

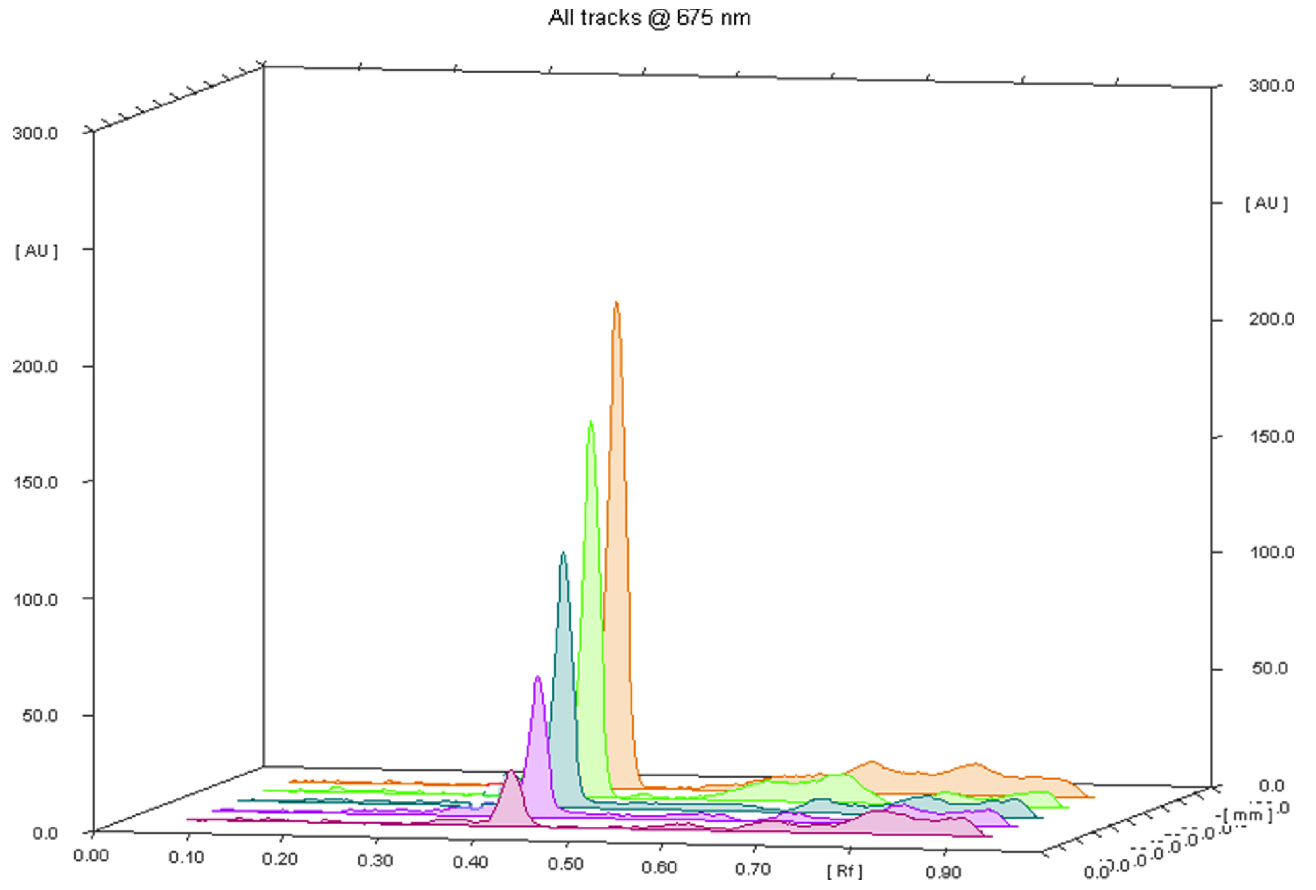


FIGURE 3. Three-dimensional overlay of HPTLC densitograms of calibration spots of tacrolimus.

TABLE 1
Linear Regression Data for the Calibration Curves ($n$ = 5)

| Range (ng/spot) | $r^2 \pm SD$ | Slope ± *SD* | Intercept ± *SD* |
|---|---|---|---|
| 100–800 | .9989 ± 0.0012 | 5.431 ± 0.1465 | 148.033 ± 10.5013 |

densitograms of the calibration spots of tacrolimus at 675 nm. The regression data as shown in Table 1 reveal a good linear relationship over the concentration range studied demonstrating its suitability for analysis. No significant difference was observed in the slopes of standard curves (ANOVA, $p > .05$).

*Accuracy*

Accuracy of an analytical method is the closeness of test results to true value. It was determined by the application of analytical procedure to recovery studies, where known amount of standard is spiked in preanalyzed samples solutions. Results of accuracy studies from excipient matrix were shown in Table 2; recovery values demonstrated the accuracy of the method in the desired range.

*Precision*

The precision of an analytical method expresses the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Intra-day precision refers to the use of analytical procedure within a laboratory over a short period of time using the same operator with the same equipment, whereas inter-day precision involves estimation of variations in analysis when a method is used within a laboratory on different days, by different analysts. The results obtained are shown in Table 3. In all instances, %*RSD* values were less than 5% confirming the precision of the method.

*Repeatability*

Ten-microliter aliquots of samples containing 100, 400, and 800 ng of tacrolimus were analyzed according to proposed method. In order to control scanner parameters, i.e., repeatability of measurement of peak area, one spot was analyzed without changing position of plate ($n$ = 7). By spotting and analyzing the same amount several times ($n$ = 7), precision of automatic spotting device and uniformity of derivatization technique were evaluated. For both parameters,

TABLE 2
Recovery Studies ($n$ = 6)

| Formulation | Amount of Drug Analyzed (ng) | Amount of Drug Added (ng) | Theoretical Concentration (ng) | Total Amount of Drug Analyzed (ng) | % Recovery ± *SD* |
|---|---|---|---|---|---|
| F1 | 300 | 240 (80%) | 540 | 535.16 | 99.10 ± 1.02 |
| | 300 | 300 (100%) | 600 | 602.09 | 100.34 ± 0.98 |
| | 300 | 360 (120%) | 660 | 649.86 | 98.46 ± 0.67 |
| | | | | %Average recovery ± *SD* | 99.3 ± 0.89 |
| F2 | 300 | 240 (80%) | 540 | 541.24 | 100.23 ± 1.60 |
| | 300 | 300 (100%) | 600 | 590.876 | 98.47 ± 0.58 |
| | 300 | 360 (120%) | 660 | 653.987 | 99.08 ± 0.31 |
| | | | | %Average recovery ± *SD* | 99.26 ± 0.83 |
| Marketed capsules | 300 | 240 (80%) | 540 | 532.3432 | 98.58 ± 1.04 |
| | 300 | 300 (100%) | 600 | 601.5432 | 100.25 ± 0.64 |
| | 300 | 360 (120%) | 660 | 650.7734 | 98.62 ± 0.37 |
| | | | | %Average recovery ± *SD* | 99.15 ± 0.68 |

TABLE 3
Intra and Inter-Precision Studies ($n$ = 5)

| Amount of Drug Spotted (ng) | Amount of Drug Detected (ng, mean ± *SD*) | %*RSD* |
|---|---|---|
| Intra-day ($n$ = 5) | | |
| 100 | 99.16 ± 4.68 | 4.92 |
| 400 | 401.01 ± 10.03 | 2.56 |
| 800 | 897.17 ± 15.79 | 1.98 |
| Inter-day ($n$ = 5) | | |
| 100 | 99.53 ± 3.13 | 3.35 |
| 400 | 398.44 ± 14.74 | 3.71 |
| 800 | 798.95 ± 18.31 | 2.29 |

%*RSD* was consistently less than 5% (Table 4), which was well below the instrumental specifications, ensuring repeatability of developed method as well as proper functioning of the HPTLC system.

*Robustness*

The low values of %*RSD* (Table 5) obtained after introducing small deliberate changes in the developed HPTLC method confirmed the robustness of the method.

## Application of Developed Method

*Determination of Equilibrium Solubility*

Tacrolimus has very low water solubility (5–8 μg/mL) (Akashi et al., 1996), which limits the development of new pharmaceutical formulations aimed to improve its delivery. Therefore, solubility studies were performed to identify suitable oily phases, surfactants, and cosurfactants that are generally employed in commercially available parenteral and oral products (Table 6). In spite of being lipophilic in nature (partition coefficient >40,000), tacrolimus exhibited very low solubility in parenterally acceptable oily phase such as soybean oil, lipoid MCT, isopropyl myristate, and miglyols. It was found that only ethyl oleate exhibited good solubility for tacrolimus.

TABLE 4
Repeatability Studies ($n$ = 7)

| Parameters | Amount of Drug Detected (ng, mean ± *SD*) | | |
|---|---|---|---|
| Amount of Tacrolimus Spotted (ng) | 100 | 400 | 800 |
| Measurement of peak area[a] | 98.63 ± 2.77 | 403.92 ± 9.09 | 789.98 ± 20.36 |
| %*RSD* | 2.81 | 2.25 | 2.58 |
| Sample application and derivatization technique[b] | 101.27 ± 1.41 | 397.69 ± 12.23 | 795.71 ± 23.37 |
| %*RSD* | 1.39 | 3.07 | 2.94 |

[a]One spot is scanned eight times.
[b]Eight spots scanned once.

TABLE 5
Robustness of Method ($n$ = 3)

| Parameters | Amount Detected (ng, mean ± *SD*) | %*RSD* |
|---|---|---|
| Mobile phase composition: 6.1:3.9:0.1 | 405.02 ± 10.09 | 2.49 |
| Mobile phase composition: 5.9:4.1:0.1 | 399.11 ± 13.24 | 2.31 |
| Mobile phase volume: 8 mL | 403.76 ± 14.82 | 2.67 |
| Mobile phase volume: 8 mL | 394.54 ± 13.29 | 1.36 |
| Chamber saturation time: 15 min | 397.89 ± 6.99 | 1.75 |
| Chamber saturation time: 25 min | 410.6 ± 7.89 | 1.92 |
| Solvent migration distance: 58 mm | 407.11 ± 8.56 | 2.10 |
| Solvent migration distance: 62 mm | 403.11 ± 10.36 | 2.57 |

The drug exhibited good solubility in Tween 80, Solutol HS-15 (surfactants), Propylene glycol, and Glycofurol (cosurfactants). Among the various orally acceptable excipients tried, the modified or hydrolyzed vegetable oily phases such as Capryol 90, Lauroglycol 90, Capmul MCM C8/10, and Capmul MCM C8 exhibited good solubilization potential for tacrolimus. Cremophore-EL amongst surfactants and Carbitol as a cosurfactant were found to solubilize maximum amount of tacrolimus.

*Determination of pH-Solubility Profile*

An understanding of pH-solubility profile of a drug candidate is regarded as one of the most important aspects of preformulation testing for poorly soluble compounds and has been found to be useful for both oral and parenteral formulation development. For example, gastrointestinal tract exhibits a pH in the range of 1.2–7.5, and the solubility profile of the drug can help in predicting the absorption behavior of that drug molecule in the gastrointestinal tract. The study clearly indicated that tacrolimus has poor aqueous solubility and it does not depend on pH of the medium (Table 6). This behavior may be attributed to the neutral nature of tacrolimus.

*Analysis of Tacrolimus in Formulations*

A single spot at $R_f$ = 0.40 was observed in the chromatogram of tacrolimus. No interference from the excipients present in the marketed capsule formulation (Pangraf™ 5 mg) was observed. Analysis of tacrolimus capsules showed a drug content of 4.98 ± 0.12 mg. The applicability of the method was verified by the determination of tacrolimus in two self-microemulsifying formulations (developed in-house), and no interference from the excipient matrix was observed. The tacrolimus content of the developed and the marketed formulations was found to be within the limits (±5% of the theoretical value) and are mentioned in Table 7. The low %*RSD* value indicated the suitability of this method for routine analysis of tacrolimus in various formulations.

TABLE 6
Solubility of Tacrolimus in Various Excipients and Buffers

| Excipients | Solubility[a] | Excipients | Solubility |
|---|---|---|---|
| *Oily phases* | | *Cosurfactants* | |
| Akomed E | 12.28 ± 1.15 | Benzyl alcohol | 42.63 ± 3.82 |
| Akoline MCM | 25.45 ± 2.21 | Carbitol | 58.69 ± 3.94 |
| Capmul MCM C8 | 32.19 ± 1.02 | Glycofurol | 22.48 ± 1.02 |
| Capmul MCM C8/10 | 28.69 ± 2.38 | Labrafil M 1944 S | 06.77 ± 0.69 |
| Capryol 90 | 34.44 ± 0.74 | Labrafac CC | 04.23 ± 1.01 |
| Ethyl oleate | 08.79 ± 1.69 | Imwitor 742 | 15.69 ± 1.75 |
| Isopropyl myristate | 00.75 ± 0.86 | Plurol Oleique | 05.08 ± 0.69 |
| Lauroglycol 90 | 33.45 ± 1.12 | Labrasol | 09.63 ± 0.82 |
| Lipoid MCT | 02.16 ± 1.11 | PEG 200 | 35.66 ± 2.79 |
| Maisine 35-1 | 06.97 ± 1.49 | PEG 300 | 32.89 ± 4.39 |
| Miglyol 810 N | 01.65 ± 1.29 | PEG 400 | 39.52 ± 3.64 |
| Miglyol 812 N | 02.10 ± 0.56 | Propylene glycol | 37.88 ± 4. 21 |
| Peceol | 07.79 ± 1.34 | Propylene glycol | 37.88 ± 4. 21 |
| Soybean oil | 01.39 ± 0.59 | Triacetin | 17.17 ± 2.21 |
| *Surfactants* | | *Aqueous phases*[c] | |
| Cremophor RH 40 | 20.32 ± 3.57 | 0.9% NaCl solution | 06.23 ± 2.46 |
| Cremophor-EL | 25.02 ± 1.16 | 5% Dextrose solution | 05.94 ± 2.53 |
| Gelucire 44/14[b] | 12.41 ± 0.35 | Buffer pH 1.2 | 06.81 ± 3.26 |
| Gelucire 55/13[b] | 11.22 ± 0.23 | Buffer pH 3.5 | 06.02 ± 3.17 |
| Lutrol F 68[b] | 13.45 ± 1.21 | Buffer pH 4.5 | 05.72 ± 2.11 |
| Lutrol F 127[b] | 09.41 ± 0.41 | Buffer pH 6.8 | 06.80 ± 3.14 |
| MYS-40[b] | 11.35 ± 0.96 | Buffer pH 7.4 | 07.11 ± 2.32 |
| Solutol HS-15 | 09.60 ± 0.89 | Buffer pH 9.0 | 07.02 ± 2.23 |
| Tween 20 | 12.76 ± 1.04 | Buffer pH 10.6 | 06.29 ± 1.69 |
| Tween 80 | 14.45 ± 2.19 | Purified water | 07.32 ± 2.59 |

[a]Data expressed as mg/g, mean ± *SD*, $n$ = 3.
[b]10% (wt/wt) surfactant solutions.
[c]Data expressed as µg/mL, mean ± *SD*, $n$ = 3.

TABLE 7
Content of Tacrolimus in Various Formulations

| Formulation | Label Claim (mg) | Amount Found (mg, mean ± *SD*) | %*RSD* |
|---|---|---|---|
| Pangraf™ capsules | 5 | 4.98 ± 0.12 | 2.40 |
| F1 | 5 | 4.95 ± 0.09 | 1.82 |
| F2 | 5 | 4.92 ± 0.11 | 2.23 |

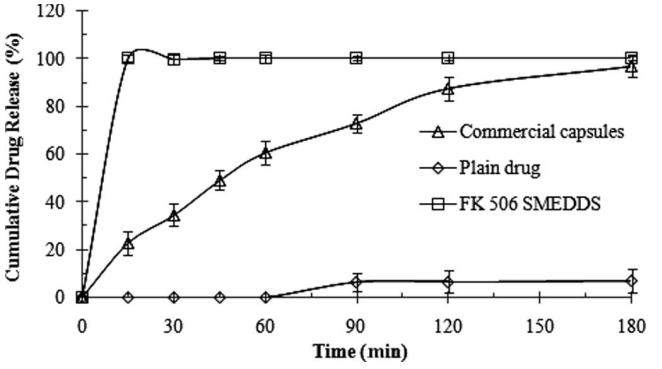


FIGURE 4. Dissolution profiles of tacrolimus from various formulations in buffer pH 1.2, data expressed as mean ± *SD*, *n* = 3.

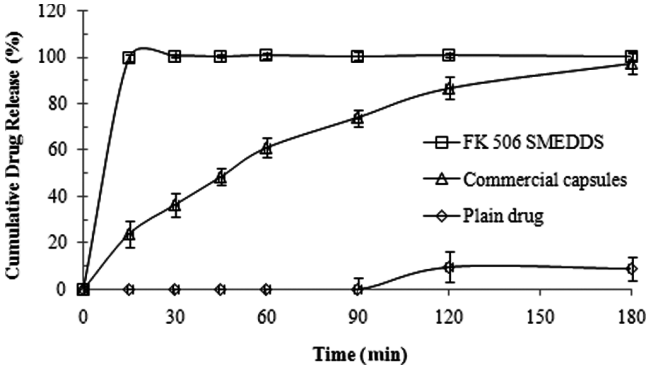


FIGURE 5. Dissolution profiles of tacrolimus from various formulations in buffer pH 6.8, data expressed as mean ± *SD*, *n* = 3.

### *In Vitro Dissolution Profile Tacrolimus Formulation*

In vitro dissolution profiles of formulation F1, commercial formulation, and plain drug in buffer pH 1.2 and 6.8 are presented in Figures 4 and 5, respectively. Formulation F1 was found to exhibit a release of 100% drug within 15 min in both dissolution media. Pangraf capsules required 2 h to release 85% of tacrolimus, whereas less than 10% of drug was released in 3 h from plain drug. It was also evident that release of tacrolimus from self-microemulsifying formulation was independent of pH of the dissolution medium and was consistent with the results of pH-solubility profile study.

## CONCLUSION

A new HPTLC method has been developed for the identification and quantification of tacrolimus. Low cost of ingredients, faster speed, and satisfactory precision and accuracy are the main features of this method. Method was successfully validated as per ICH guidelines and statistical analysis proves that method is sensitive, specific, and repeatable. It can be conveniently employed for routine quality control analysis of tacrolimus as bulk drug in marketed capsules and self-microemulsifying formulations without any interference from excipients. The method was also applied for the estimation of equilibrium solubility of tacrolimus in various excipients and dissolution studies.

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## REFERENCES

Akashi, T., Nefuji, T., Yoshida, M., & Hosoda, J. (1996). Quantitative determination of tautomeric FK506 by reversed-phase liquid chromatography. *J. Pharm. Biomed. Anal., 14*, 339–346.

Alak, A. M., Moy, S., & Menard, S. (1997). An HPLC/MS/MS assay for Tacrolimus in patient blood samples correlation with results of an ELISA assay. *J. Pharm. Biomed. Anal., 16*, 7–13.

Arima, H., Yunomae, K., Miyake, K., & Uekama, K. (2001). Comparative studies of the enhancing effects of cyclodextrins on solubility and oral bioavailability of Tacrolimus in rats. *J. Pharm. Sci., 90*, 690–701.

Beysens, A. J., Wijnen, R. M. H., & Beuman, G. H. (1991). Tacrolimus: Monitoring in plasma or in whole blood? *Transplant. Proc., 23*, 2745–2747.

Borhade, V. B. (2006). *Study of specialized emulsions for drug delivery*. Thesis, for the degree of master of pharmaceutical sciences, University of Mumbai, Mumbai, India.

Borhade, V., Nair, H., & Hegde, D. (2008). Design and evaluation of self-microemulsifying drug delivery system (SMEDDS) of Tacrolimus. *AAPS PharmSciTech., 9*, 13–21.

Christine, E. A., & Susan, E. (2004). Clinical pharmacokinetics and pharmacodynamics of FK506 in solid organ transplantation. *Clin. Pharmacokinet., 3*, 623–653.

Deters, M., Kaever, V., & Kirchner, G. I. (2003). Liquid chromatography/mass spectrometry for therapeutic drug monitoring of immunosuppressants. *Anal. Chim. Acta, 492*, 133–145.

Dutta, S., Mezei, M., & Lee, T. D. (1998). Liposomal Tacrolimus and intestinal drug concentration. *Transplant. Proc., 30*, 2651–2652.

Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., & Kohsaka, M. (1987). FK506, a novel immunosuppressant isolated from a streptomyces I. Fermentation, isolation and physico-chemical and biological characteristics. *J. Antibiot., 42*, 1249–1255.

Koch, W. L. (1998). Erythromycin. In K. Florey (Ed.), *Analytical profiles of drug substances* (Vol. 8, pp. 159–177). London: Academic Press.

McAlister, V. C., Keshavamurthy, M., & Lee, T. D. G. (1999). Oral delivery of liposomal tacrolimus: Increased efficacy and reduced toxicity. *Transplant. Proc., 31*, 1110–1113.

Namiki, Y., Fujiwara, A., Kihara, N., Koda, S., & Hane, K. (1995). Factors affecting tautomeric phenomenon of a novel potent immunosuppressant (FK506) on the design for injectable formulation. *Drug Dev. Ind. Pharm., 21*, 809–822.

Shapiro, R. J. (1999). Tacrolimus in solid organ transplantation: An update. *Transplant. Proc., 31*, 2203–2205.

Spencer, C. M., Goa, K. L., & Gillis, J. C. (1997). Tacrolimus: An update of its pharmacology and clinical efficacy in the management of organ transplantation. *Drugs, 54*, 925–975.

Takada, K., Hashi, M., Yoshikawa, H., & Muranishi, S. (1990). Determination of a novel potent immunosuppreesant (FK-506) in rat serum and lymph by high-performance liquid chromatography with chemiluminescence detection. *J. Chromatog., 530*, 212–218.

Tamura, K., Kobayashi, M., & Rnhimoto, K. (1987). A highly sensitive method to assay Tacrolimus levels in plasma. *Transplant. Proc., 19*(Suppl. 6), 23–29.

Wallemacq, P. E., & Reding, R. (1993). FK-506, a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects. *Clin. Chem., 39*, 2219–2228.

Yamashita, K., Nakate, T., Okimoto, K., Ohike, A., Tokunaga, Y., Ibuki, R., Higaki, K., & Kimura, T. (2003). Establishment of new preparation method for solid dispersion formulation of tacrolimus. *Int. J. Pharm., 267*, 79–91.