DRUG FORMULATIONS AND CLINICAL METHODS

Validation of a Liquid Chromatographic Method for Determination of Tacrolimus in Pharmaceutical Dosage Forms

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An accurate, simple, and reproducible liquid chromatographic method was developed and validated for the determination of tacrolimus in capsules. The analysis is performed at room temperature on a reversed-phase C18 column with UV detection at 210 nm. The mobile phase is methanol–water (90 + 10) at a constant flow rate of 0.8 mL/min. The method was validated in terms of linearity, precision, accuracy, and specificity by forced decomposition of tacrolimus, using acid, base, water, hydrogen peroxide, heat, and light. The response was linear in the range of 0.09–0.24 mg/mL (r^2 = 0.9997). The relative standard deviation values for intra- and interday precision studies were 1.28 and 2.91%, respectively. Recoveries ranged from 98.06 to 102.52%.

Tacrolimus (TCR) is a potent macrolide immunosuppressant derived from *Streptomyces tsukubaensis* and has actions similar to those of cyclosporin. It is used to prevent or reverse rejection in patients receiving organ transplants. It has been tried in a few patients with refractory autoimmune or immune-mediated disorders.

The usual capsule doses are 0.5, 1.0, and 5.0 mg anhydrous TCR. In this study we developed and validated a new chromatographic method for quantitation of TCR in capsules. The method was validated by following the analytical performance parameters suggested by the International Conference on Harmonization (ICH; 1).

Most of the analytical techniques described for TCR in the literature are based on the liquid chromatographic determination of this drug in human blood (2–11). The aim of our investigation was to develop and validate a liquid chromatography (LC) method for the determination of TCR in the presence of its degradation products in pharmaceutical dosage forms.

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Experimental

Standard

The TCR standard was obtained from Inter-Chemical Ltd. (Wan Chai, Hong Kong) with a purity of 96,70%, calculated with reference to the dried substance.

Sample

A commercial capsule formulation was studied. Its composition was 1 mg TCR in a matrix of hydroxypropyl methylcellulose, croscarmellose sodium, lactose, magnesium stearate, and titanium dioxide.

Reagents

(**a**) *Methanol*.—LC grade (Sintorgan S.A., **CITY???**, Argentina). (Solvents were filtered through a 0.45 m membrane and degassed.)

(**b**) *High-performance LC grade water*.—Millipore® Milli-Q system **(AUTHOR: CITY/STATE/COUNTRY??)**.

Chromatographic Conditions and Instrumentation

The LC system consisted of a dual-piston reciprocating Spectra-Physics **(AUTHOR: CITY/STATE/COUNTRY??)** Model ISO Chrom. LC pump, a UV-Vis Hewlett-Packard Model 1050 detector, a Hewlett-Packard Series 3395 integrator, **(AUTHOR: CITY/STATE/ COUNTRY??)** and a Rheodyne Model 7125 injector. The analytical column was a reversed-phase C18 column (Ace, Aberdeen, Scotland; 250 × 4.6 mm, 5 m). The mobile phase was methanol–water (90 + 10) pumped at a constant flow rate of 0.8 mL/min. UV detection was at 210 nm. The liquid chromatograph was operated at ambient temperature. The injection volume was 20 L. Under these conditions, the retention time (t_R) of TCR was approximately 6 min.

Preparation of Solutions

Solutions were prepared on a weight basis, and volumetric flasks were used as suitable containers to minimize solvent evaporation.

Table 1. Selectivity: conditions for degradation of TCR

Condition	Time, h	TCR, %	RRT of degradation products[a]
Acid (0.1N HCl, reflux)	0.5	48.2	0.70, 0.82, 0.90, 1.27, 1.63
Base (0.05N NaOH, reflux)	0.5	34.3	0.69, 0.80, 1.73
Hydrogen peroxide, 30% (reflux) **(AUTHOR: TEXT SAYS 15%)**	0.5	—	0.56, 0.85
Water (reflux)	0.5	94.5	0.55, 1.62
Heat dry, 110°C (solution)	24	91.2	0.54
Heat dry, 110°C (solid)	24	100.0	0.47, 0.55
Daylight exposure	24	98.0	0.53

[a] RRT = Relative retention time.

Standard Solution

A stock solution of TCR was prepared at a concentration of 1.5 mg/mL by dissolving the appropriated amount of TCR standard in mobile phase. The standard solution was obtained by diluting the stock solution with mobile phase to obtain a solution containing TCR at 150 g/mL.

Sample Preparation

The contents of 20 capsules were placed in a mortar, and an amount of powder equivalent to 3.75 mg TCR was added to a 25 mL volumetric flask; 20 mL mobile phase was added, and the flask was placed in an ultrasonic bath for 5 min. The contents of the flask were then diluted to 25 mL with mobile phase, thoroughly mixed, and filtered through a 0.2 m nylon membrane, 25 mm disposable filter (Cat. No. Y02025WPH; icroclar, **(AUTHOR: IS THIS THE CO. NAME?)** Buenos Aires, Argentina).

Method Validation

(**a**) *System suitability test*.—Relative standard deviation (RSD) values for the peak area, tailing factor, and retention time were the chromatographic parameters selected for the system suitability test (12).

(**b**) *Specificity*.—Forced degradation studies were performed to evaluate the specificity of the method. Degraded samples were prepared by refluxing the TCR stock solution at 1.5 mg/mL with acid (0.1N HCl), base (0.05N NaOH), water, and 15% **(AUTHOR: TABLE 1 SHOWS 30%)** hydrogen peroxide and refluxing for ≥30 min. Drug samples were subjected to thermal degradation (either in the solid state or in solution in an open container in an oven at 110°C for 24 h and photochemical degradation (a solution of the drug was transferred to a container and exposed to daylight for 24 h). After degradation, samples were allowed to cool at room temperature and diluted, if necessary, to the same concentration as that of the standard solution, after neutralization. After degradation, samples were analyzed by using the methodology and the chromatographic conditions described.

(**c**) *Linearity*.—A stock solution of TCR at 300 g/mL was prepared in a 50 mL volumetric flask by dissolving 15 mg TCR standard in mobile phase. Appropriate volumes of the stock solution were diluted with mobile phase to obtain solutions containing TCR at 90.0, 120.0, 150.0, 180.0, and 240.0 g/mL. Each solution was injected in triplicate into the chromatograph.

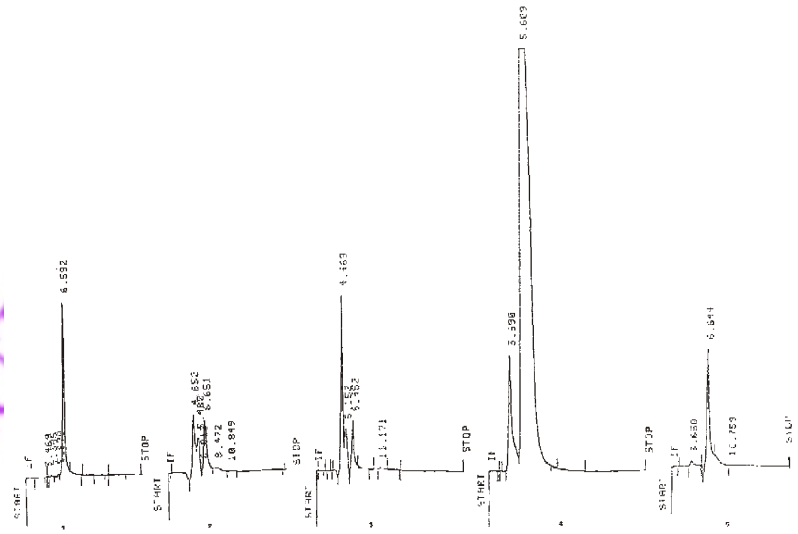

Figure 1. Chromatograms of TCR obtained during degradation tests using reflux conditions: (1) standard; (2) acid hydrolysis (0.1N HCl, reflux 0.5 h); (3) alkaline hydrolysis (0.05 N NaOH, reflux 0.5 h); (4) oxidation (30% hydrogen peroxide, reflux 0.5 h); (5) hydrolysis (water, reflux 0.5 h).

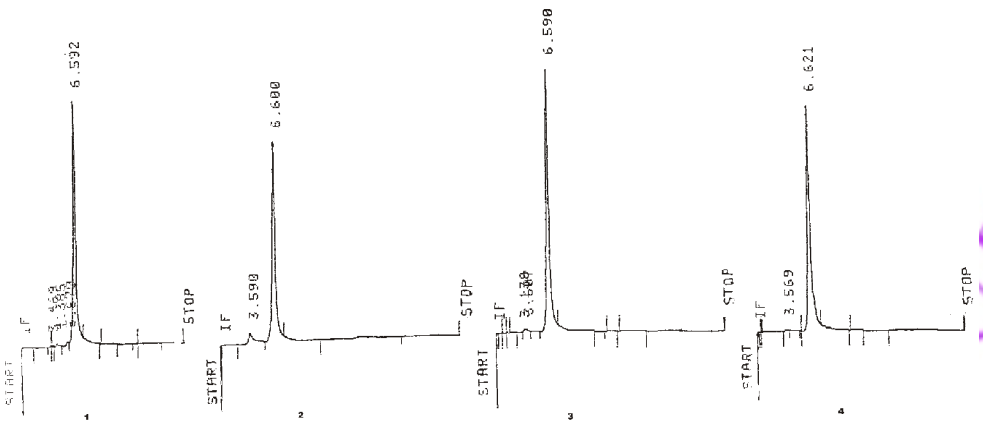

Figure 2. Chromatograms of TCR obtained during thermal and photochemical degradation tests: (1) standard; (2) heat dry, 110 C (solution, 24 h); (3) heat dry, 110 C (solid, 24 h); (4) daylight exposure (24 h).

(**d**) *Precision*.—Repeatability was calculated by analyzing 6 samples. Intermediate precision was assessed by comparing the results obtained for analyses of 6 samples, prepared by 2 different analysts on 2 different days.

(**e**) *Accuracy*.—Recovery was studied at TCR concentration levels of 80, 100, and 120% (3 samples each). The contents of 20 capsules from the same lot of a commercial formulation were placed in a mortar. The amounts of TCR recovered in relation to the results obtained in the intermediate precision study were calculated.

(**f**) *Robustness*.—Robustness was established by changing the proportions of the components in the mobile phase.

(**g**) *Limits of detection (LOD) and quantitation (LOQ)*.—Serial dilutions of TCR in mobile phase were made to obtain concentrations ranging from 1.55 to 4.65 g/mL.

Table 2. Linearity data for determination of TCR

TCR, % of nominal value	TCR injected, g	Average peak area response	RSD, %[a]
60	1.824	17858133.3	0.80
80	2.432	23551088.0	0.37
100	3.040	29176150.7	0.37
120	3.648	34532981.3	0.69
160	4.864	46373322.7	0.33
Slope[b]	9344690.1	249826.2	0.96
Intercept[c]	754162.9	831959.4	

[a] RSD = Relative standard deviation.
[b] Confidence limits of the slope (P = 0.05).
[c] Confidence limits of the intercept (P = 0.05).

Results and Discussion

The reversed-phase LC method described in this paper was developed to provide a rapid quality control determination of TCR in capsules. The method was validated according to ICH guidelines. The method uses a simple mobile phase. All samples were analyzed by using the chromatographic conditions described.

No evidence of interactive degradation products was found during the evaluation. However, analyses for TCR showed evidence of degradation products after the degradation treatments. Degradation was indicated in the stressed sample by a decrease in the expected concentration of the drug and increased levels of degradation products. The results of the stress study are presented in Table 1. Selectivity was demonstrated, showing that TCR was free of interference from degradation products, and that no interference from the sample excipients was observed at the detection wavelength; thus, the proposed method can be used in a stability assay (Figures 1 and 2). **TBL 01; FIGS 01, 02**

The assay range of the method was set at 80–120% of the label claim of the finished product. The linearity of the detector responses was determined by preparing calibration graphs. The linearity of the peak response versus concentration was studied from 0.09 to 0.24 mg/mL. The representative linear equation was 9 344 690x + 754163 with a standard error ($S_{x,y}$) of 210500, and the correlation coefficient (r) **(AUTHOR: ABSTRACT SAYS r^2)** was 0.9999; the intercept was not significantly different from zero (P = 0.05; Table 2). **TBL 02**

The precision and accuracy of the assay were demonstrated. Precision is usually expressed as the RSD of a series of measurements. In the study of the precision of the instrumental system, an RSD of 0.7% was obtained for the TCR peak area. In all of these cases, the RSD obtained was <1.5%, the limit set for the precision of the instrumental system, showing that the equipment used for the study

Table 3. Precision of the method for determination of TCR

	Analyst 1		Analyst 2	
Sample	TCR found, mg/capsule	RSD, %	TCR found, mg/capsule	RSD, %
1	1.061	0.10	1.127	0.22
2	1.081	0.10	1.048	0.22
3	1.045	0.10	1.044	0.22
4	1.049	0.10	1.072	0.22
5	1.058	0.10	1.084	0.22
6	1.047	0.10	1.045	0.22
Mean	1.057	1.22	1.070	3.02

Table 4. Recovery of TCR

TCR, % of nominal value	TCR added, mg	TCR found, mg	Recovery, %	Average recovery, %[a]	RSD, %
80	0.744	0.751	100.93	101.96	0.80
	0.732	0.750	102.43		
	0.754	0.773	102.52		
100	0.969	0.980	101.19	100.80	2.21
	0.924	0.913	98.89		
	0.965	0.987	102.32		
120	1.125	1.122	99.76	99.24	2.45
	1.172	1.149	98.06		
	1.152	1.151	99.91		
Mean[b]				100.67	1.57

[a] $n = 3$.
[b] $n = 9$.

operated correctly for the developed method and produced highly repetitive results.

The intraday precision was evaluated by analyses of the samples on 2 different days by 2 different analysts. The results were reported both individually and as an average. For the precision assays, the results were as follows: mean values of 1.057 and 1.070 mg per capsule and RSD values of 1.22 and 3.02%. A *t*-test comparing 2 samples with 95% confidence for 10 degrees of freedom showed that the results were not significantly different ($t_{n-2,\ :0.05} = 2.23$; Table 3). TBL 03

The results obtained in the accuracy study (recovery test) with 9 samples of 1 commercial formulation ($n = 3$ for 80, 100, and 120%) indicated that the mean recovery was 100.67%. The RSD was 1.57%. The experimental *t*-value of the percent recovery value was 1.280, which is far below the 2.306 established for the tabulated *t*-value (95% level of probability, 8 degrees of freedom; Table 4). TBL 04

The effects of the proportions of the components of the mobile phase on resolution and retention time are shown in Table 5. Retention time was significantly affected. TBL 05

The LOD attained as $LOD_{(k=3.3)} = k \times S_a/b$, where b is the slope of the calibration graph and S_a is the standard deviation (SD) of the *y*-intercepts of a regression line, was found to be 25 ng for an injection volume of 20 L. The LOQ was also attained as $LOQ_{(k=10)} = k \times S_a/b$, and was found to be 76 ng for an injection volume of 20 L (Table 6).

Table 5. Robustness of the method for determination of TCR

Mobile phase	RT, min	RS[b]
Methanol–water (95 + 5)	6.5	0.2
Methanol–water (85 + 15)	7.8	0.9

[a] RT = Retention time of TCR.
[b] RS = Resolution. **(AUTHOR: CORRECT DEFINITION??)**

Conclusions

The reversed-phase LC method developed in this study is precise, accurate, and specific. The method was completely validated, showing satisfactory data for all the method

Table 6. Analytical data for the determination of LOD and LOQ

TCR injected, mg	Average peak area response	SD
0.031	130 535.3	52268
0.062	938 382.0	34192
0.093	1367 254.7	14266
Slope[a]	–612 934.5 ± 74 112.6	
Intercept[b]	71 577.3 ± 4963.1	
Correlation coefficient	–0.9996	

[a] Confidence limits of the slope ($P = 0.05$).
[b] Confidence limits of the intercept ($P = 0.05$).

validation parameters tested. The developed method can be used for the routine analysis of production samples and also to check the stability of bulk samples of TCR.

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