

Determination of tacrolimus in pharmaceutical formulations by validated spectrophotometric methods

Böer, T.M.^{1*}; Marques, M.R.¹; Cardoso, S.G.²

¹Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, UFSM, Santa Maria, RS, Brasil.
²Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, UFSC, Florianópolis, SC, Brasil.

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ABSTRACT

Two simple, rapid and sensitive spectrophotometric methods were developed for the determination of tacrolimus in pharmaceutical dosage forms. The methods, based on the sulphuric acid reaction and on the iodine charge-transfer reaction, gave absorption peaks at 295 nm and 365 nm, respectively. The calibration curves were linear in the concentration range of 30-55 $\mu\text{g mL}^{-1}$ for the sulphuric acid method ($r^2=0.9999$) and 5-10 $\mu\text{g mL}^{-1}$ ($r^2=0.9999$) for the charge-transfer method. The specificity was assessed, showing that there was no interference from the excipients. The accuracy of both of the methods was higher than 99.44%, with a bias lower than 2%, and high precision was also demonstrated. The limits of quantitation for the two methods were 30 $\mu\text{g mL}^{-1}$ and 5 $\mu\text{g mL}^{-1}$. The proposed methods were applied to the determination of tacrolimus in capsule dosage forms, and the results compared statistically with the validated reversed-phase liquid chromatography (RP-LC) method, showing significant correlation ($p<0.05$) and demonstrating either method to be an excellent alternative to LC. The application of these simple methods to routine quality control analysis of pharmaceuticals could contribute to their safety and therapeutic efficacy.

Keywords: tacrolimus; sulphuric acid reaction; iodine charge-transfer; spectrophotometry; pharmaceutical formulations.

INTRODUCTION

Tacrolimus is a macrolide lactone with alpha, beta-diacetamide hemiacetal incorporated in the 23-membered ring structure, as shown in Figure 1. It is extracted from *Streptomyces tsukubaensis* and has potent immunosuppressive activity, being used clinically in the prevention of organ transplant rejection, such as liver, kidney, heart, pancreas and bone marrow. The drug is also indicated for the treatment of atopic dermatitis, eczema, psoriasis and vitiligo. It is insoluble in water, slightly

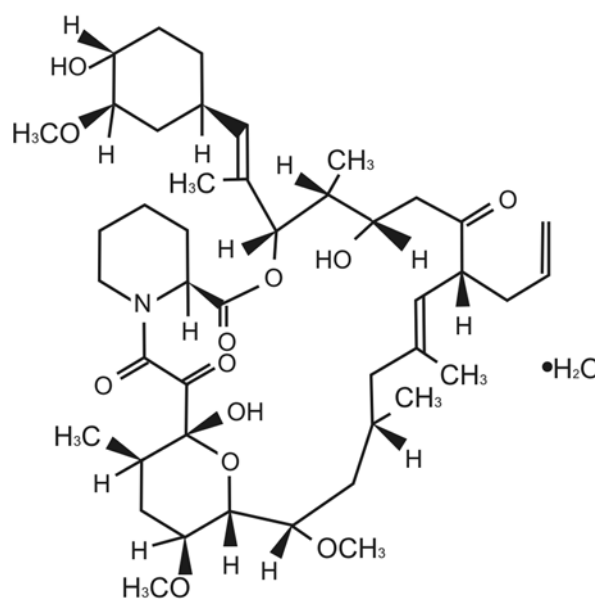


Figure 1. Chemical structure of tacrolimus.

soluble in saturated hydrocarbons, and highly soluble in lipids and other organic solvents (Kino et al., 1987; Goto et al., 1991). Pharmaceutical dosage forms such as capsules, injection and ointment are available for clinical use.

In the 1980s, the introduction of the potent immunosuppressive drugs cyclosporin A and tacrolimus reduced the incidence of rejection episodes after solid organ transplantation. Their chemical structures are different, but the mechanisms of action are similar: inhibiting the transcription of the first signal for T-lymphocyte activation. However, clinical studies demonstrated that tacrolimus is a potent alternative to cyclosporin A, due to its lower nephrotoxicity and reversible neurotoxicity (Garcia et al., 2004).

A validated method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), with detection by positive-ion electrospray ionization (ESI+), was used to determine the pharmacokinetic profile of tacrolimus in biological fluids (Taylor et al., 1996). A micellar electrokinetic capillary chromatography method was developed for the quantitation of tacrolimus in pharmaceutical samples, with detection at 214 nm,

using 20 mM phosphate buffer (pH 7.5), 50 mM sodium dodecylsulphate and methanol as organic modifier for the background electrolyte solution (Tripodi et al., 2001). A reversed-phase liquid chromatography (RP-LC) method with a C_{18} column was developed and validated for the assay of tacrolimus and its tautomeric forms in capsules, with UV detection at 220 nm (Namiki et al., 1995). The quantitation of tacrolimus and testing of the uniformity of its content in ointment were performed on a TSK ODS-80 column with isocratic flow of the mobile phases (Xu et al., 2005). The isocratic RP-LC method was also validated for the quantitation of tacrolimus in capsules, using a C_{18} column and UV detection at 210 nm with a linear range of 0.09-0.24 mg mL⁻¹ (Moyano et al., 2006). The effect of diluents and medical devices on the stability of tacrolimus in parenteral solutions was also evaluated by the RP-LC (C_{18}) method with detection at 214 nm (Taormina et al., 1992). The same method was used to test the stability of tacrolimus in an extemporaneous compounded oral liquid formulation, prepared by mixing the contents of commercially available capsules containing 5 mg with appropriate diluents, and the stability of the drug was confirmed in both glass and plastic amber bottles (Jacobson et al., 1997). Moreover, sensitive and accurate spectrophotometric methods have been described for the determination of sparfloxacin in tablets with visible light (Marona & Schapoval, 2001) and of lomefloxacin and gatifloxacin in tablets and raw material with UV detection (Gomes & Salgado, 2005; Salgado & Oliveira, 2005). The spectrophotometric method based on the sulphuric acid reaction was developed and applied to the identification and quantitation of macrolide antibiotics, giving reproducible results (Danielson et al., 1993). The reaction of n-donor drugs with the σ -electron acceptor iodine to form a charge-transfer complex followed by triiodide ion-pair formation has been described (Bebawy et al., 1999). The iodine was shown to react with basic molecules of electron donor solvents such as the nitrogen of acetonitrile, producing the I_3^- ion, but despite this interaction, acetonitrile was shown to be an appropriate solvent for the complex formation, without interference in the validation (Moustafa, 2000). The spectrophotometric methods are robust and easy to use and hence are widely used for the quantitation of drugs in formulations when there is no interference from the excipients. At present, tacrolimus is not described in any Pharmacopoeia.

The aim of the present study was to develop and validate simple, fast, selective and economical spectrophotometric methods for the routine analysis of tacrolimus in both industrial-scale and small-scale manipulated pharmaceutical formulations, so as to improve and accelerate quality control, and to ensure the therapeutic effectiveness of the medicinal products.

MATERIAL AND METHODS

Instrumentation

A calibrated Spectronic Genesis 2 UV-VIS spectrophotometer (Milton Roy G., USA), with a fixed slit width (2 nm) and a 10 mm quartz cell, was used to record UV spectra and measure absorbance.

Chemicals

The tacrolimus reference substance (assigned purity, 98.8%) was obtained from SP-Pharma (São Paulo, Brazil). Commercial batches of tacrolimus capsules Prograf® (JANSSEN-CILAG, São Paulo, Brazil) with a stated content of 1 mg were used within their shelf life. Concentrated sulphuric acid and acetonitrile were obtained from Tedia (Fairfield, USA) and iodine from Merck (Darmstadt, Germany).

Solutions

Preparation of reference substance solution

The stock solution was prepared by accurately weighing 10 mg of tacrolimus reference substance, which was transferred to a 20 mL volumetric flask, dissolved and diluted to volume with acetonitrile, to obtain a concentration of 500 μ g mL⁻¹.

Sample preparation

To prepare the sample solution twenty capsules of tacrolimus were individually weighed to obtain their mean weight, and then an amount of the finely crushed powder equivalent to 5 mg was transferred to a 10 mL volumetric flask, dissolved and made up to the mark with acetonitrile, to obtain a concentration of 500 μ g mL⁻¹. The solution was filtered through quantitative filter paper (Schleicher & Schuell) and stored at 2-8°C.

Methodology

Sulphuric acid spectrophotometric method

Aliquots of 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1 mL of reference stock solution and sample solution, at 500 μ g mL⁻¹, were transferred to 10 mL volumetric flasks and 400 μ L of concentrated sulphuric acid added, and the mixture was diluted to volume with acetonitrile. The absorbance was measured at 295 nm against a solvent blank. The solutions were stable for 10 minutes.

Iodine charge-transfer spectrophotometric method

Aliquots of 100, 120, 140, 160, 180 and 200 μ L of reference stock solution and sample solution at a concentration of 500 μ g mL⁻¹ were transferred to 10 mL volumetric flasks, 4 mL of 0.01M iodine was added and the volume completed

with acetonitrile. The absorbance of the solutions was measured at 365 nm against a blank prepared by mixing the solvents. The solution was stable for two hours.

Validation of the methods

The methods developed were validated using samples of pharmaceutical capsules nominally containing 1 mg of tacrolimus, according to the label, by determining the following parameters: specificity, linearity, precision, accuracy, limit of quantitation (LOQ) and robustness, following the International Conference on Harmonisation (ICH Q2(R1)) guidelines (ICH, 2005).

Specificity

The specificity of the methods was assessed by evaluating the interference of the excipients of the pharmaceutical formulation. A solution containing only placebo (in-house mixture of all the capsule excipients) was prepared by the same procedure as the sample and the UV spectrum of this solution was recorded in the range of 200–400 nm, to reveal possible interfering bands at 295 and 365 nm, for the sulphuric acid and iodine charge-transfer spectrophotometric methods, respectively.

Linearity

Linearity was determined by constructing three independent analytical curves, each one with six calibration points for tacrolimus, with the concentrations 30, 35, 40, 45, 50 and 55 $\mu\text{g mL}^{-1}$ and 5, 6, 7, 8, 9 and 10 $\mu\text{g mL}^{-1}$, for the sulphuric acid and charge-transfer spectrophotometric methods, respectively. The absorbance values were plotted against the respective concentrations of tacrolimus to obtain the analytical curves. The results were subjected to regression analysis by the least squares method to calculate the calibration equation and correlation coefficient.

Precision and accuracy

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by performing six determinations of the same concentration of tacrolimus, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days). The accuracy was evaluated by comparing the results of the proposed methods with those of the published chromatographic procedure (Taormina et al., 1992).

Limit of quantitation

The LOQ was calculated for each method from the slope and the standard deviation of the intercept of the mean of three calibration curves, determined by a linear regression model defined in ICH Q2 (R1) guidelines (ICH, 2005).

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability in routine analysis. The robustness was determined by analyzing the same samples under a variety of conditions of the method parameters, such as the volumes of the sulphuric acid and iodine. To assess the stability of sample solutions of tacrolimus, the samples tested were held at 4 °C and 25 °C for 48 hours.

RESULTS

Sulphuric acid spectrophotometric method

After the reaction with concentrated sulphuric acid, the absorption spectrum showed highest absorbance peaks at 220 nm, related to the excipients, and at 295 nm, the latter being selected for the analytical studies, as shown in Figure 2. The reaction was optimized by adding several volumes of sulphuric acid, from 100 to 500 μL , to the tacrolimus solution, and measuring the absorbances at various times from 0-60 minutes. The maximum absorbance was achieved

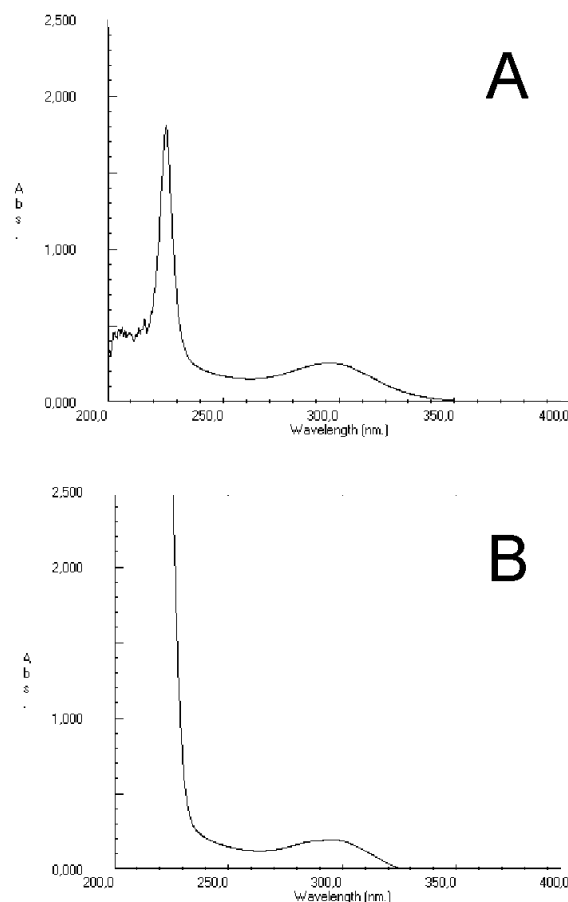


Figure 2. UV spectrum of sulphuric acid reaction with (A) tacrolimus solution and (B) capsules, at 40 $\mu\text{g mL}^{-1}$ in acetonitrile at 25 °C.

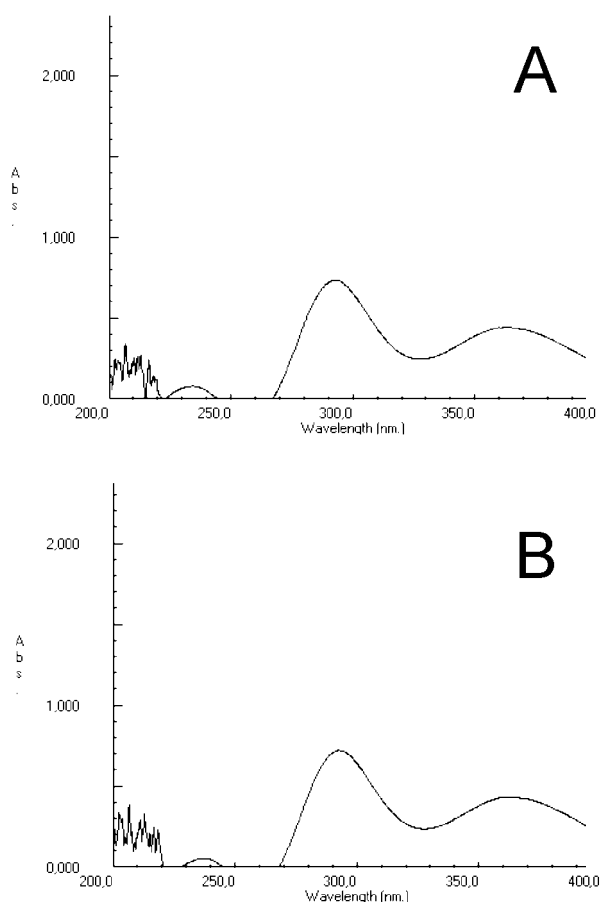


Figure 3. UV spectrum of iodine (0.01M) charge-transfer reaction with (A) tacrolimus solution and (B) capsules, at $8 \mu\text{g mL}^{-1}$ in acetonitrile at 25°C .

with $400 \mu\text{L}$ of concentrated sulphuric acid and the reaction was stable for 10 minutes at 25°C .

Iodine charge- transfer spectrophotometric method

The reaction between tacrolimus and iodine resulted in a complex which showed two absorption maxima, at 300 nm and 365 nm. Interference of the excipients was detected at 285 nm and the measurements were carried out at 365 nm, as shown in Figure 3. The experimental conditions tested showed that the addition of 4 mL of 0.01M iodine was most suitable, producing a reproducible and stable complex, for at least two hours.

Method Validation

The tests of specificity of the methods showed no interference from the excipients (lactose, hypromellose, sodium croscarmellose and magnesium stearate) of the pharmaceutical formulation at 295 and 365 nm, as shown in Figures 4 and 5, thus demonstrating that the proposed methods are specific for the analysis of tacrolimus at these wavelengths.

The linearity curves were defined, for the sulphuric

acid and iodine charge-transfer spectrophotometric methods respectively, by the following equations: $y = (0.01 \pm 0.0001)x - (0.0712 \pm 0.003)$ and $y = (0.0988 \pm 0.012)x - (0.1435 \pm 0.015)$, where x is concentration and y is absorbance. The correlation coefficients calculated for both methods were $r^2=0.9999$, indicating the linearity of the analytical curves with very significant regression ($p<0.05$). The LOQs were calculated by the calibration equations generated by using the mean values of the three

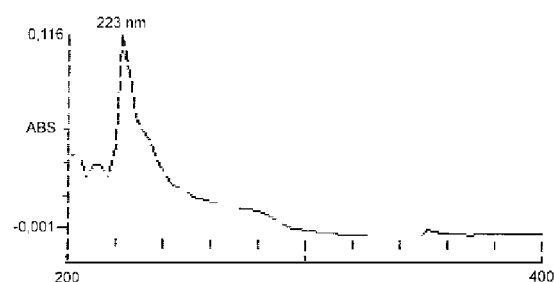


Figure 4. UV spectrum of sulphuric acid reaction with the capsule excipients dissolved in acetonitrile at 25°C .

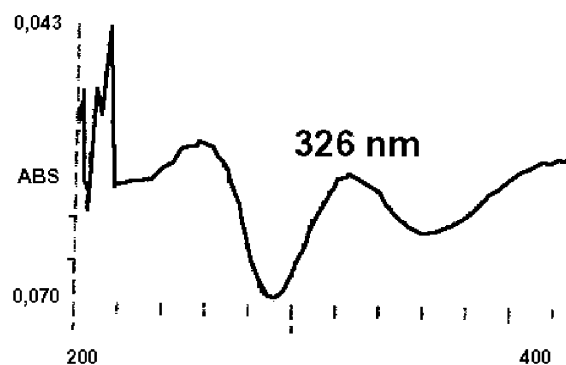


Figure 5. UV spectrum of iodine (0.01M) charge-transfer reaction with the capsule excipients dissolved in acetonitrile at 25°C .

independent analytical curves, giving values of 30 and $5 \mu\text{g mL}^{-1}$, respectively, for the sulphuric acid and iodine charge-transfer spectrophotometric methods.

The precision, evaluated as the repeatability of the method, was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of $40 \mu\text{g mL}^{-1}$ and $8 \mu\text{g mL}^{-1}$ for the sulphuric acid and iodine charge-transfer spectrophotometric methods, respectively. The RSD was $<0.2\%$ for both of the methods, as shown in Table 1. The intermediate precision was also assessed, by analyzing the samples of the pharmaceutical formulations on three different days (inter-day); the mean values obtained were 99.3 and 99.7%, with RSD of 0.2 and 0.1%, respectively.

The results obtained over the experimental range of the selected variables evaluated in the robustness assessment show that there were no significant changes in the method

Table 1 - Intra-day and inter-day precision data for sulphuric acid and iodine charge-transfer spectrophotometric methods and RP-LC method for tacrolimus capsule dosage forms.

Method	Intra-day ^a		Inter-day ^b	
	% ± SD ^c	%RSD ^d	% ± SD ^c	%RSD ^d
Sulphuric acid method	99.4 ± 0.13	0.1	99.3 ± 0.11	0.2
Iodine charge-transfer method	99.6 ± 0.15	0.2	99.7 ± 0.07	0.1
RP-LC	98.2 ± 0.5	0.1	-	-

^a Mean of six determinations

^b Mean of three determinations

^c SD = Standard deviation

^d RSD = Relative standard deviation

when reasonable modifications were made in the experimental conditions, thus showing the method to be robust. The stability of the reaction solutions was demonstrated as 10 min and two hours, respectively. The solutions of tacrolimus were shown to be stable at 4°C and 25°C for 48 hours.

The validated spectrophotometric methods were applied to the determination of tacrolimus in the same batches of capsule dosage forms, without prior separation of the excipients of the formulation, giving, for the means of the replicates, recovery

values exceeding 99.44%, as shown in Table 2. The results were compared by ANOVA with a validated RP-LC method described elsewhere (Taormina et al., 1992), demonstrating good accuracy, with mean values less than 1.44% higher for the spectrophotometric methods, with significant correlation ($p < 0.05$).

Table 2 - Analysis of variance (ANOVA) for the sulphuric acid and iodine charge-transfer methods relative to the reversed-phased (RP-LC) method.

Method ^a	Sulphuric acid	RP-LC ^a	Iodine method ^b
Mean content found (%)	99.44	98.21	99.65
Standard deviation	0.13	0.52	0.16
Variance	0.017	0.27	0.026
ANOVA	3.9 (7.71) ^c		13.55 (21.20) ^c

^a Mean of three determinations

^b Mean of six determinations

^c Critical F value at P = 0.05

DISCUSSION

Tacrolimus exhibits low UV absorption, and as a consequence the sensitivity achieved by conventional UV-spectrophotometric methods is not suitable for quantitative analysis. The sulphuric acid reaction, previously studied for macrolide antibiotics (Danielson et al., 1993; Han et al., 2006), was validated for the identification and quantitation of the macrolide tacrolimus.

For the iodine charge-transfer spectrophotometric method, as demonstrated in the literature (Moustafa, 2000), acetonitrile is a suitable solvent for the formation of the complex between tacrolimus and iodine, with validation of the procedure. Hence, iodine was diluted in acetonitrile for the determination of tacrolimus in pharmaceutical formulations.

The results of the validation studies show that the two spectrophotometric methods are simple, fast, specific and precise for the determination of tacrolimus in capsules. Therefore, the methods proposed represent reliable and easily-performed alternatives to the more time-consuming RP-LC, requiring minimal sample preparation and widely available laboratory facilities, for the quantitative analysis of tacrolimus in both industrially produced and manually prepared pharmaceutical formulations, contributing to the improvement of quality control and therapeutic efficacy.

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RESUMO

Determinação de tacrolimus em formulações farmacêuticas por métodos espectrofotométricos validados

Desenvolveram-se dois métodos espectrofotométricos, simples e sensíveis para a determinação de tacrolimus em formas farmacêuticas. Os métodos baseados na reação com ácido sulfúrico e na transferência de carga com iodo, apresentaram absorção máxima a 295 e 365 nm, respectivamente. As curvas de calibração foram lineares na faixa de concentração de 30-55 µg/mL para o método do ácido sulfúrico ($r^2=0,999$) e 5-10 µg/mL ($r^2=0,999$), para o complexo de transferência de carga com iodo. Avaliou-se a especificidade demonstrando ausência de interferência dos excipientes. Avaliou-se também a precisão e exatidão para ambos os métodos com resultados superiores a 99,44%, e erro menor do que 2%. Os limites de quantificação foram 30 µg/mL e 5 µg/mL. Os métodos foram aplicados para a determinação

de tacrolimus em cápsulas e os resultados comparados estatisticamente com o método por cromatografia líquida em fase reversa, demonstrando correlação significativa ($p<0,05$). A aplicação desses métodos alternativos no controle da qualidade contribuirá para garantir a segurança e eficácia terapêutica dos produtos farmacêuticos.

Palavras-chave: tacrolimus; ácido sulfúrico; transferência de carga com iodo; espectrofotometria; formulações farmacêuticas.

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