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Electroreduction of Tacrolimus as Immunosuppressant agent at Hanging Mercury Drop Electrode: Application to Determination in Pharmaceutical Formulation

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ABSTRACT

The adsorptive behavior of the immunosuppressive agent tacrolimus was studied by cyclic and differential-pulse voltammetry on a hanging mercury drop electrode (HMDE). The drug was accumulated at HMDE and two well-defined peak currents were obtained at -1353 and -1417 mV *vs*. SCE (saturated KCl) in borate buffer (pH 10.0) + 0.1 KCl solution. A voltammetric procedure was developed for the determination of tacrolimus using differential-pulse adsorptive stripping voltammetry (DPAdSV). The optimum working conditions for determination of the drug were established. The analysis of tacrolimus in pharmaceutical dosage forms was carried out satisfactorily.

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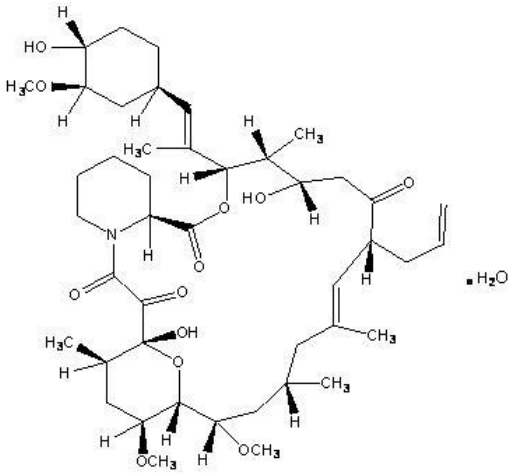
Introduction

Tacrolimus (Scheme 1), a hydrophobic macrocyclic lactone produced by *Streptomyces tsukubaensis*, is used as both primary and rescue therapy for heart, renal and lung-transplant recipients [1]. Tacrolimus has a narrow therapeutic window and exhibits concentration-related efficacy and toxicity with considerable inter- and intra-patient variability in pharmacokinetics [2, 3]. Because of these factors frequent therapeutic drug monitoring of whole-blood trough concentrations is recommended for optimization of tacrolimus therapy [4]. It is suggest that a trough level therapeutic window of 5 to 20 ng ml^{-1} may optimize efficacy and minimize the side effects [5]. Up to now a number of analytical techniques have been developed for the quantitative determination of tacrolimus. Many of these methods rely on the use of high performance liquid chromatography/tandem mass spectrometry (HPLC-MSMS or LC-MS) technique [6-13]. Although, the LC-MS method provides remarkable low detection limits, it is limited because of the high instrumentation and initial costs. Some authors have attempted to use the immunoassay methodology with antibodies that recognize the tacolimus molecule [14, 15]. However, antibody-based assays have been shown to overestimate tacrolimus concentrations due to cross-reactivity with tacrolimus metabolites [16, 17].
Electroanalytical techniques have some important advantages including speed, high sensitivity, relative simplicity and low costs compared to other techniques. Adsorptive stripping voltammetry is a highly sensitive technique for the analysis of organic compounds which can be accumulated at the surface of hanging mercury drop electrode (HMDE) and afterwards stripped off by applying a potential scan [18]. To our knowledge, there is no previous electrochemical data concerning the electrochemical behavior of tacrolimus on HMDE. The aim of the work presented here was to develop a sensitive electroanalytical procedure for the determination of tacrolimus in pharmaceutical formulations based on its adsorption and reduction on HMDE.

Experimental Section

Apparatus

All voltammograms were recorded using a Metrohm multifunction instrument model 693 VA processor equipped with a 694 VA stand. A VA-Database software version 2.2 installed on a Pentium-IV computer was used for storing and processing data. Measurements were carried out with a hanging mercury drop electrode (HMDE), (size: 6), in a three-electrode arrangement. The auxiliary electrode was a wire of platinum with a considerably larger surface area than that of HMDE. A saturated calomel electrode (SCE) was used as reference electrode. Solutions were deoxygenated with high-purity nitrogen for 5 min prior to each experiment**.** All measurements were carried out at room temperature under the nitrogen atmosphere. A Metrohm-692 digital pH-meter was used for pH measurements.



Scheme 1. Structure of tacrolimus

Chemicals

All chemicals used were of analytical reagent grade and were obtained from Merck. Doubly distilled deionized water was used for all electrochemical experiments. Tacrolimus powder was obtained from Sigma and was used without further purification. A 100 µg ml^{-1} stock solution of tacrolimus was prepared in methanol and stored in the dark at 4°C. More dilute solutions were prepared daily from this stock by accurate dilution with methanol.

General procedure

A 10 ml aliquot of background electrolyte was introduced into the voltammetric cell and the solution was purged with nitrogen for 300s.Then, the accumulation potential (0.0 mV) was applied to the working electrode for 50s while the solution was

stirred continuously at 700 r.p.m. Then stirring was stopped and after 5 s equilibrium time, a negative potential sweep was started and the resulting voltammogram was recorded as background electrolyte voltammogram. An aliquot of the standard tacrolimus solution was introduced into the cell with a micropipette and the adsorptive voltammugram was repeated using a new mercury drop as before.

Preparation of capsule assay solutions

The contents of ten capsules (Prograf®) were completely removed from shells and mixed. An amount equivalent to a single capsule was weighed and dissolved in 10 ml of pure methanol and vortexed for two minuets. After the undissolved excipients have settled down, a more diluted solution was prepared in methanol using an aliquot of the clear supernatant. A 25 µl aliquot of this solution was introduced into the voltammetric cell containing 10 ml borate buffer (pH 10.0) + 0.1M KCl. Differential pulse adsorptive stripping voltammograms were recorded as described in the general procedure section. The content of the drug in capsules was determined referring to the regression equation of the adsorptive stripping voltammetry.

Results and Discussion

Voltammetric behavior of tacrolimus

To elucidate the electrode reaction of tacrolimus, a cyclic voltammogram at hanging mercury drop electrode was recorded at different pH and at different scan rates. As an example, Figure 1A shows the cyclic voltammogram of 250 ng ml^{-1} tacrolimus in Britton-Robinson buffer (pH 10.0) at a scan rate of 50 mVs^{-1}. As seen, two cathodic waves are observed when potential is scanned in the negative directions.

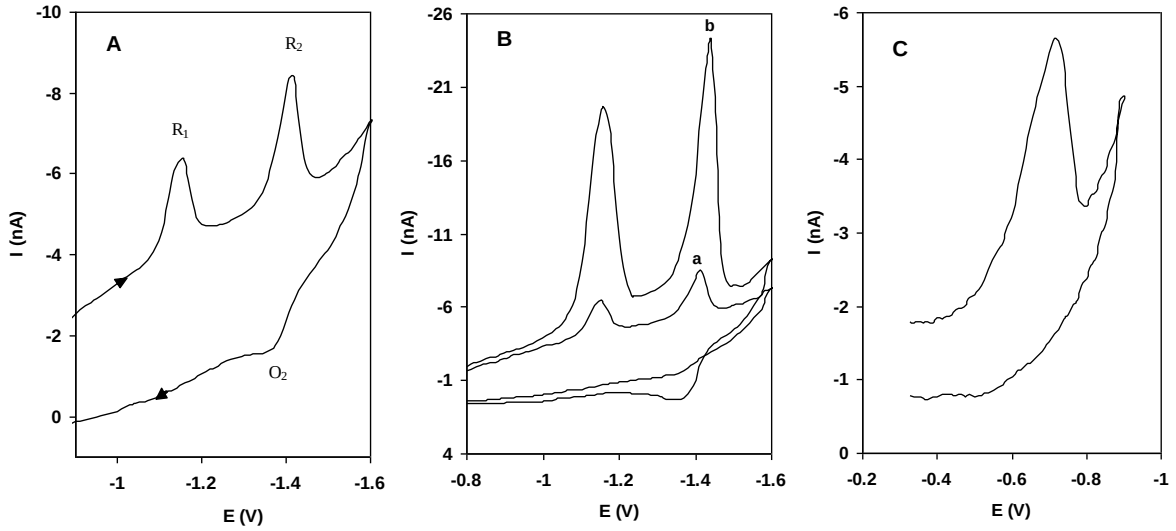


Fig. 1. (A) (A) Cyclic voltammogram of 250 ng ml^{-1} tacrolimus in Britton-Robinson buffer pH 10.0, (B) cyclic voltammogram of the same solution a) without accumulation time and b) with 20 s accumulation at 0.0 mV. (C) Cyclic voltammogram of 1.0 µg ml^{-1} tacrolimus in Britton-Robinson buffer pH 2.0.

First peak is observed at -1150 mV (R_1) and second one at -1417 mV (R_2). On scanning in the positive direction, only one anodic peak is observed at -1353 mV (O_2). The difference between peak potentials of R_2 and O_2 is about 64 mV. These observations confirm the irreversibility of the first electrode process and quasi- reversibility of the R_2/O_2 redox couple. The adsorptive characteristic of the reduction waves is also demonstrated by recording the cyclic voltammograms when accumulation parameters (time and potential) are applied to the working electrode. As can be seen in Figure 1B after applying a short accumulation time of 20 s at 0.0 mV the peak currents were largely increased. This indicates the adsorptive character of the drug onto the mercury surface during the accumulation step. The effect of potential scan rate (v) on the peak currents was also examined. A linear relationship was observed between log i_p and log v for first (R_1) and second (R_2) reductive peaks with the slops of 0.91 (R^2> 0.999)

and 0.89 (R^2> 0.998), respectively (Figure 2). These slopes are close to the theoretically expected value of 1.0 for an ideal reaction of surface species.

It should be noted that, the cyclic voltammogram of the drug in acidic media shows only one irreversible reduction process at -700 mV (Figure 1C). At this medium the plot of log i_p vs. log v is linear with the slope of 0.74 (R^2> 0.997). This indicates the reduction process is controlled by both adsorption and diffusion in acidic media.

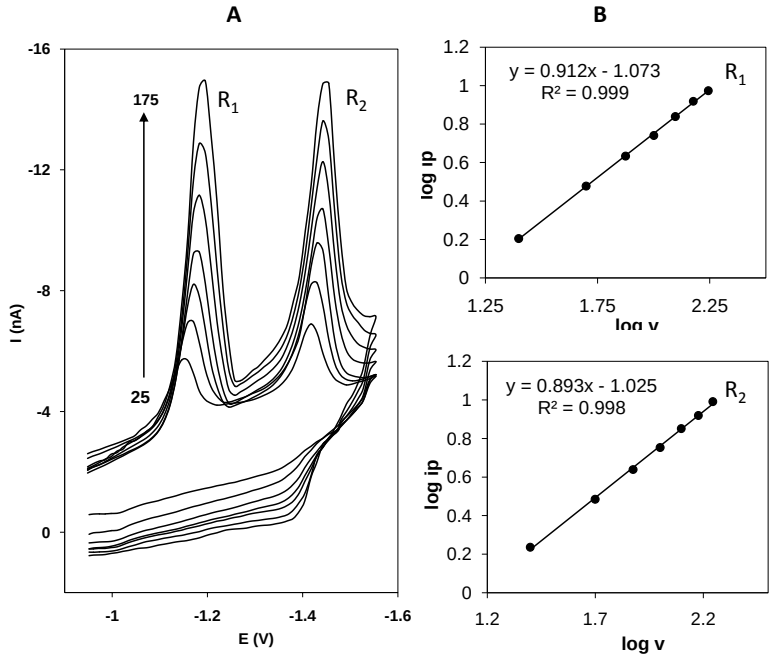


Fig. 2. A) Cyclic voltammograms of 250 ng ml^{-1} of tacrolimus in Britton-Robinson buffer pH 10.0 recorded at different scan rates, and B) corresponding log ip vs. log v plots for R_1 and R_2 waves.

Optimization of parameters affecting the adsorptive stripping response

Effect of pH and supporting electrolyte

The nature and pH of the supporting electrolyte are some of the most important factors which strongly influence the cathodic reduction and adsorption processes. Figure 3 shows the differential-pulse adsorptive stripping voltammograms for 25 ng ml^{-1} tacrolimus in Briton-Robinson buffer at different pHs. As seen, tacrolimus exhibits a single cathodic peak in acidic media (pH<6), while two well-defined peaks can be observed at higher pH values (pH>6). No considerable changes were observed in both cathodic currents at pHs higher than 10.0. Thus, this pH was chosen as analytical pH for further measurements. As can be seen from Figure 3 the second reduction current is about twice than that obtained for the first reduction peak. In order to enhance the sensitivity of the method the second reduction peak was chosen as analytical signal and optimization of further parameters was performed using this peak.

The effect of various supporting electrolytes such as KCl, NaCl, KNO_3, $NaNO_3$, and $NaClO_4$ on the stripping signal was tested. Both the peak height and the peak shape were taken into consideration when choosing the supporting electrolyte. The results showed that, the best signal could be obtained in a solution containing 0.1 M of KCl. Also, diverse buffer solutions were tested for their suitability in the determination of tacrolimus, as follows: phosphate, borate, carbonate, Britton-Robinson, and ammonia buffers. The most suitable buffer system for the determination of the drug was found to be 0.04 M borate buffer of pH=10. Therefore, the optimal conditions for studying the adsorptive stripping voltammetry of tacrolimus is a mixture of 0.04 M borate buffer (pH=10) and 0.1 M KCl.

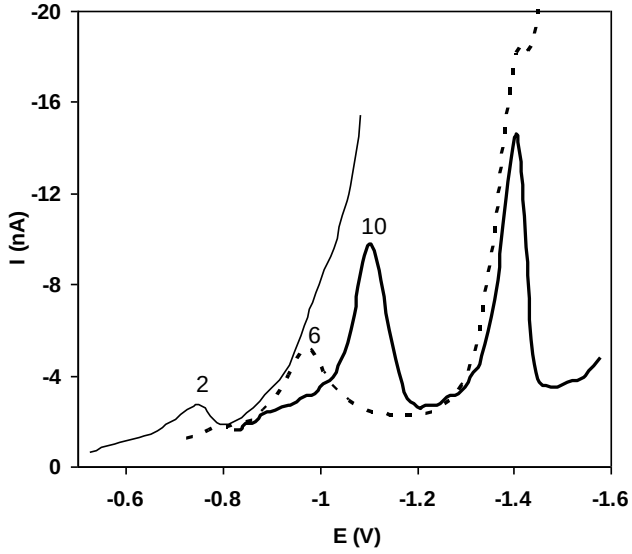


Fig. 3. Differential pulse voltammograms of 25 ng ml^{-1} tacrolimus at different pHs. t_{acc} = 30 s, E_{acc} = 0.0 mV, v = 40 mV s^{-1}. Numbers indicate the pH of the solutions.

Pulse amplitude, pulse width and scan rate

The influence of some instrumental parameters known to affect the differential pulse voltammograms, viz. pulse amplitude, pulse width and scan rate were studied. During the study, each variable was changed while the other two were kept constant. The variables of interest were studied over the ranges of 25-100 mV for pulse amplitude, 30-90 ms for pulse width and 10-50 mV s^{-1} for scan rate. To acquire voltammograms of relatively high sensitivity and well-shaped waves with relatively a narrow peak width, values of 50 mV, 0.03 s and 40 mV s^{-1} were chosen for pulse amplitude, pulse width and scan rate, respectively.

Accumulation potential

The influence of the accumulation potential (E_{acc}) on the peak height was studied over the range 50 to -300 mV for three tacrolimus concentration levels in the selected supporting electrolyte and an accumulation period of 50 s. The observed peak currents were plotted against applied potential and are shown in Figure 4. As seen, the peak currents reach the maximum values at an accumulation potential of 0.0 mV. The adsorbed species are most probably neutral molecules of the drug and the maximum of peak current is reached in the potential range of zero charge of mercury electrode [19, 20]. Therefore, a pre-concentration potential of 0.0 mV was used throughout the present study.

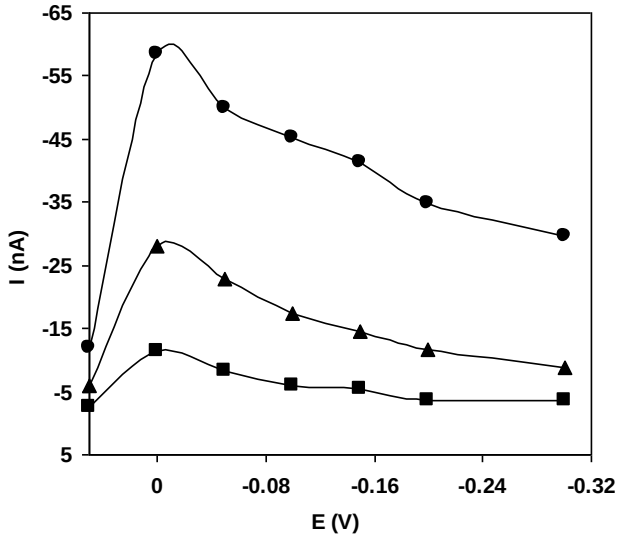


Fig. 4. Effect of accumulation potential on the peak current of 10 ng ml^{-1} (■), 30 ng ml^{-1} (▲) and 60 ng ml^{-1} (●) of tacrolimus in borate buffer (pH 10) + 0.1 M KCl (t_{acc} = 50 s).

Accumulation time

The effect of accumulation time was studied for different concentrations of tacrolimus (10, 30, and 60 ng ml^{-1}). As can be seen from Figure 5, the peak current values depend strongly on the accumulation time, suggesting an effective adsorption of tacrolimus on the HMDE. The peak current increases with increasing accumulation time, indicating the enhancement of tacrolimus concentration at the electrode surface. As the accumulation time increases, the peak current tends to level off, illustrating that the adsorptive equilibrium is reached. The plot of peak current versus the accumulation time (t_{acc}) for 10 ng ml^{-1} was linear up to approximately 90 s, while for 30 and 60 ng ml^{-1} tacrolimus, the response was linear up to 80 and 90 s. Thus, an accumulation time of choice should be dictated by the sensitivity needed.

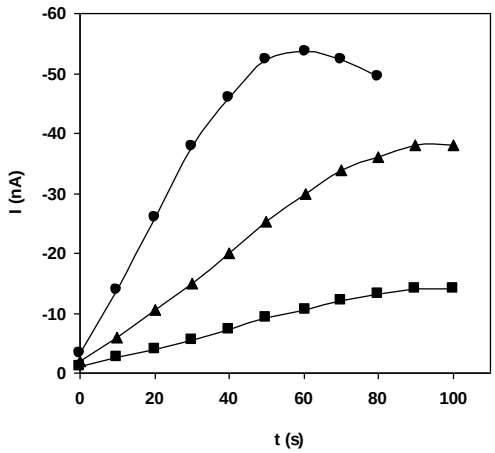


Figure 5. Effect of accumulation time on the peak current of 10 ng ml^{-1} (■), 30 ng ml^{-1} (▲) and 60 ng ml^{-1} (●) of tacrolimus in borate buffer (pH 10) + 0.1 M KCl (E_{acc} = 0.0 mV).

Figures of merit

Under the selected conditions, the reduction peak current of tacrolimus yields well-defined concentration dependence. Voltammograms for solutions of increasing tacrolimus concentration were obtained after 90, 50, and 0 s accumulations, and in all cases sharp and well-defined peaks were observed. The calibration equations, limits of detection (LOD) and other parameters are listed in Table 1. The values of LOD were calculated as (3*s*/*b*), where *s* is the standard deviation of the arithmetic average of 8 voltammograms of blank solution performed at the same potential as tacrolimus and *b* is the slope of the calibration curve. Figure 6, shows voltammograms and corresponding calibration curve of tacrolimus after applying an accumulation time of 50 s. Eight successive measurements of 5 ng ml^{-1} of tacrolimus after 50 s accumulation showed the relative standard deviation of 1.0%.

Table 1. Characteristics of the calibration curves of voltammetric determination of tacrolimus

Accumulation time (s)	Regression equation	r^2 (n=16)	LOD (ng ml^{-1})
0	i_p (nA) = –0.016 *C* (ng ml^{-1}) – 1.594	0.9993	0.06
50	i_p (nA) = –0.889 *C* (ng ml^{-1}) – 2.579	0.9998	0.11
90	i_p (nA) = –1.800 *C* (ng ml^{-1}) – 2.458	0.9998	26

Interference study

The effects of some possible interfering compounds mainly as excipients were investigated. Synthetic sample solutions containing 10 ng ml^{-1} tacrolimus and different concentrations of the other compounds were tested, and the peak heights were measured. The tolerance limit was defined as the ratio of interfering to tacrolimus concentration, which gave an error less than 3.0% in the determination of tacrolimus.

Table 2 shows the maximum tolerable concentrations of the various compounds. It can be seen that glucose, ascorbic acid, sorbitol, citric acid, sucrose, lactose, and starch have no significant effect on the determination of tacrolimus even when they are present up to 250 to 1000 times the weight ratio of tacrolimus. From the data in Table 2, it can also be concluded that thiol-containing compounds such as cysteine produce a striking interference in the determination of tacrolimus because of their strong tendency toward the mercury surface.

Table 2. Interferences of some foreign species on the determination of 10 ng ml^{-1} tacrolimus

Foreign species	Tolerance limit (C_I/C_{TAC}) [a]
Starch, Lactose, Sucrose	1000
Sorbitol, Citric acid	500
Arginine	400
Glucose, Ascorbic acid	250
Alanine	100
Oxalate, carbonate	60
Lucien, Glycine	50
Histidine	40
Cysteine	0.2

[a] C_I : Interference concentration,
C_{TAC} : tacrolimus concentration

Real sample analysis

The proposed voltammetric procedure was applied to the analysis of two formulations of Prograf® capsules containing 0.5 and 1 mg drug per capsule using the calibration plot (Figure 6) after a simple sample preparation (Table 3) For 0.5 mg labeled capsules, the obtained percentage mean recoveries (%R) and the relative standard deviations (%RSD) based on the average of five replicate measurements were 98.8 and 1.42, respectively. These values were 101.0 and 1.58 for 1 mg labeled capsules, respectively.

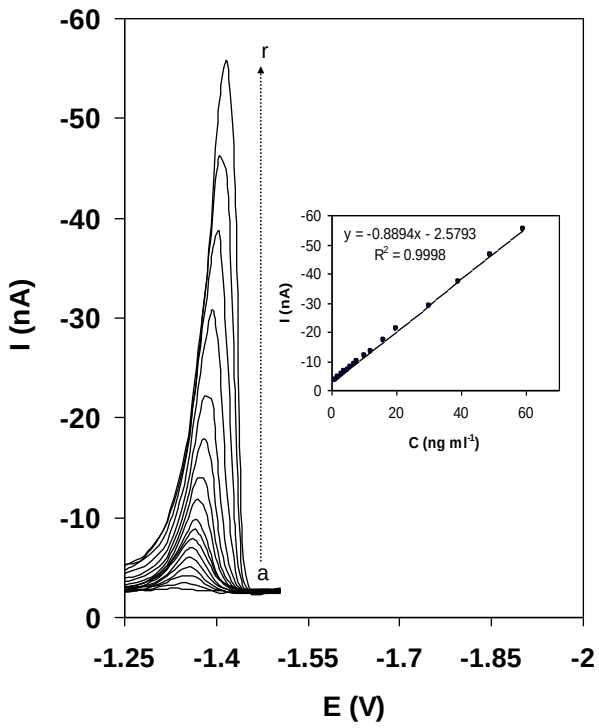


Fig. 6. Differential pulse-adsorptive stripping voltammograms for different concentrations of tacrolimus (1.0-60 ng ml^{-1}) in borate buffer (pH 10) + 0.1 M KCl (E_{acc} = 0.0 mV, t_{acc} = 50 s, ν = 40 mV s^{-1}, pulse amplitude = 50 mV, pulse width 0.03 s).

Conclusion

In this work, we have described the quantitative determination of tacrolimus using differential pulse-adsorptive stripping voltammetry on a hanging mercury drop electrode (HMDE). The proposed method was successfully applied to the determination of the drug in commercial formulations, and in general, satisfactory results were obtained. Preparation of the samples was easy and the method is cheap and fast. Therefore, this quick and simple analytical procedure is of good applicability and can be introduced to the determination of the drug in pharmaceutical dosage forms.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

Acknowledgments

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