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*Original paper*

# Tacrolimus concentrations by ELISA and LC-MS/MS

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## Abstract

Introduction. Tacrolimus has immunosuppressive effects *in vitro* through the inhibition of mixed lymphocyte reactivity and the generation of cytotoxic T cells. There is a wide variety of laboratory methods for monitoring of immunosuppressive drugs. There is a growing clinical needfor a new evaluation of these methods.

Material and Method. A total of 17 transplant patients were retrospectively analyzed. This study aimed to evaluate the concordance between ELISA and LC-MS/MS methods for measurement of tacrolimus serum trough levels.

Result. Mean tacrolimus blood concentrations in these patients ranged from 2.43-10.07 ng/ml for the ELISA and 3.1-7.6 ng/ml for the LC-MS/MS. We found a concordance between ELISA and LC-MS/MS but the inter-subject variance coefficient for the ELISA method was higher than inter-subject variance coefficient for LC-MS/MS.

Conclusion. The results point out the advantages of LC-MS/MS over ELISA in monitoring tacrolimus levels in pediatric kidney transplanted patients. The lower inter-subject variance of LC-MS/MS could result in fewer dose changes and an overall better immunosuppression control.

**Keywords** ELISA, LC-MS/MS, tacrolimus concentration.

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# Introduction

Early studies have found a good correlation between the results of tacrolimus blood concentrations determined by high-performance liquid chromatography tandem-mass spectrometry (LC-MS/MS) and enzyme-linked immuno-sorbent assay (ELISA) in solid organ transplanted patient samples (ALAK & al [1]). Another study found that the ProTrac II Tacrolimus ELISA underestimates tacrolimus concentration by ~25% on average (CAO & al [2]). However, the PRO-Trac II Tacrolimus ELISA was validated for the assessment and management of tacrolimus blood concentrations to renal transplant patients (MACFARLANE & al [3]). ELISA used to measure tacrolimus blood concentrations provided information of predictive value for managing the risk of nephrotoxicity, other toxicity, and rejection to liver transplant patients (VENKATARAMANAN & al [4]).

Recent studies used ELISA as the standard test when looking at a possible association between Tacrolimus (FK 506) levels and CMV and Polyoma BK viral loads (ZHANG & al [5]). Tacrolimus concentration using different types of ELISA was recommended to be routinely used to adjust the treatment regimen in renal transplant patients (WANG & al [6]). ELISA also yielded Tacrolimus drug concentrations similar to the atomic force microscopy values (MENOTTA & al [7]). But the emerging wide variety of laboratory methods currently available for monitoring of immunosuppressive drugs (ZHANG & al [8]) requires a new evaluation of these methods.

This study aimed to 1) evaluate time differences in tacrolimus concentrations monitored in pediatric patients after organ transplantation, 2) evaluate the concordance between ELISA and LC-MS/MS for measurement of tacrolimus concentrations.

# Materials and Methods

A total of 17 transplant patients were retrospectively analyzed. PRO-Trac II Tacrolimus ELISA was performed on DSX 4 plates ELISA processing system in the Immunological Laboratory belonging to the Emergency Hospital for Children Cluj-Napoca. Tacrolimus (FK 506) LC-MS/MS was performed in the Laboratoire CERBA belonging to the Cerba HealthCare group France for the same patient samples.

### Statistical analysis

The data was described by descriptive statistics as mean, median, interquartile interval (percentile 25% - percentile 75%), maximum and minimum for quantitative variables and frequencies for categorical variables. Because there was missing data in repeated measurements of tacrolimus concentrations, we included in the study only patients with at least 2 determinations in the studied time frame. If a patient did not have determinations for each period, these were replaced by median value of measurements.

In order to evaluate difference in tacrolimus concentrations between three time points (T1: 0-3 months, T2: 3-6 months, T3: 7-9 months), we used the Friedman and ANOVA tests .

The Bland-Altman analysis also was used to evaluate the agreement between two methods, because it is a more appropriate statistical method to compare clinical techniques that measure the same characteristic (variable). The overlap between tacrolimus concentrations measured by the 2 methods was highlighted by the Bland-Altman graph which was used to describe the absence of any systematic proportional bias. The averages of the tacrolimus concentrations determined were represented against the difference between measurements performed by ELISA and LC-MS/MS and 95% of the estimated differences are expected to lie within the limits of agreement (LoA).The bias and limits of agreement (LoA) were estimated to assess the performance of two methods, evaluating the agreement and precision between them. The inter-assay coefficients of variation (CV) for each method were also determined and compared.

For all bilateral tests a statistical significance was considered when p-values <0.05.

# Results and Discussion

*The difference in tacrolimus concentrations between three time points (T1: 0-3 months, T2: 3-6 months, T3: 6-9 months) in pediatric patients after organ transplantation:*

We included in our study 17 patients with at least 2 ELISA determinations in the periods 0-3 months, 3-6 and 6-9 months and 16 patients who had at least 2 LC-MS/MS determinations in the above-mentioned periods. The sex ratio was similar in both groups with 9/8 lot respectively in the ELISA lot and 7/9 in the LC-MS/MS lot .

We did not find any differences in the concentrations of tacrolimus determined by ELISA (Friedman test, $\chi 2$ (2) = 5.77, p = 0.063) and tacrolimus concentrations determined by LC-MS/MS (Friedman test, $\chi$ 2 0.22, p = 0.895), in either case, the distribution of tacrolimus concentrations did not show significant variations over time (Table 1).

**Table 1.** Descriptive statistics for tacrolimus concentrations (ng/ml) for each sample of determinations

| *Time points* | *Mean* | *Median(IQR*)* | *Min-max* |
|---|---|---|---|
| ***Tacrolimus blood concentrations (ng/ml ) measured by ELISA ($n_1$=17)*** | | | |
| T1[a] | 8.46 | 7.30 (5.58-8.11) | 2.43-34.20 |
| T2[b] | 7.40 | 5.54 (4.94-7.75) | 1.92-30.50 |
| T3[c] | 5.83 | 6.03 (3.40-7.32) | 1.76-11.80 |
| ***Tacrolimus blood concentrations (ng/ml) measured by LC-MS/MS ($n_2$=16)*** | | | |
| T1 | 5.03 | 4.78 (3.20-6.30) | 1.20-10.20 |
| T2 | 5.35 | 4.85 (3.50-6.28) | 2.20-15.20 |
| T3 | 5.71 | 5.45 (4.40-7.25) | 3.10-8.20 |

*IQR=interquartile range (percentile 25% - percentile 75%); [a] 0-3 months; [b] 4-6 months; [c] 7-9 months.

*The analysis of tacrolimus concentrations agreement between ELISA and LC-MS/MS*

To analyze the concordance of tacrolimus concentrations measured by the two methods, only 9 patients had determinations of tacrolimus by both methods for all three studied time points (0-3 months, 3-6 months, 6-9 months).

Mean tacrolimus concentrations in these patients range from 2.43-10.07 ng/ml for the ELISA and 3.1-7.6 ng/ml for the LC-MS/MS. Although there was an average decrease in tacrolimus concentration determined by the ELISA, these differences in tacrolimus concentration means were not statistically significant (Anova test, F (2; 16) = 1.48, p = 0.257). Regarding the concentration of tacrolimus determined by the LC-MS/MS, we did not find statistically significant differences in the tacrolimus concentration averages (Anova test, F (2; 16) = 0.09, p = 0.917) (Table 2).

**Table 2.** Descriptive statistics for tacrolimus concentrations (ng/ml) (n=9)

| | *Tacrolimusblood concentrations (ng/ml)* | | |
|---|---|---|---|
| *Time points* | *Mean* | *Median(IQR*)* | *Min-max* |
| T1[a ELISA] | 7.30 | 7.67 (4.61-10.10) | 2.43-11.90 |
| T2[b ELISA] | 6.12 | 4.94 (3.04-8.54) | 1.92-12.20 |
| T3[c ELISA] | 5.32 | 5.03 (3.40-6.73) | 2.80-8.80 |
| T1[aLC-MS/MS] | 5.58 | 5.40 (4.50-7.00) | 3.10-8.20 |
| T2[bLC-MS/MS] | 5.72 | 5.30 (4.40-6.70) | 3.20-8.40 |
| T3[cLC-MS/MS] | 5.52 | 5.50 (5.20-6.10) | 3.10-8.20 |

*IQR=interquartile range (percentile25%-percentile75%); [a] 0-3months; [b]4-6 months; [c] 7-9 months.

The regression line between the measurement bias and the differences between the measurements is within the limits of the concordance (Figure 1). We can say that the existing bias is negligible. Its trend does not differ significantly from the zero bias line. The difference in mean (bias) tacrolimus blood concentration between ELISA and LC-MS/MS was 0.64ng/ml with LoA ranging from -4.76 to 6.03.

The inter-assay coefficient of variance for the ELISA was shown to have a wider range than for LC-MS/MS (Figure 2).

Tacrolimus trough level concentration has been the standard for all solid organ post transplant monitoring. It has been shown to correlate well with AUC levels. (WONG et al [9]). Target blood concentration recommendations are different for the first year post transplant and they are dependant on the induction therapy used immediately after kidney transplantation. Studies recommend aiming for higher levels in the first 3 months (10-15 ng/ml), some authors suggest extending these targetsfor months 3-12 (5-15 ng/ml), thereafter tolerating lower levels (5-10 ng/ml) past 12 months of renal graft survival (MAYER et al [10]). Studies have shown a decrease in acute rejection episodes with higher trough levels of tacrolimus (10-15 ng/ml), the correlation being strongest when looking at the initial 3 months levels. However, this advantage comes at the expense of increased nephrotoxicity as the levels surpass the 15 ng/ml threshold (KERSHNER et al [11]). Tacrolimus has a narrow therapeutic window and there is important intra- and inter-subject pharmacokinetic variability, both of these points underline the clinical imperative for accurate drug level monitoring throughout the treatment (FILLER et al [12]). Better control of tacrolimus levels in the first 3 months after transplant has been linked to better long term graft survival in pediatric renal transplanted patients (LARKINS et al [13]).

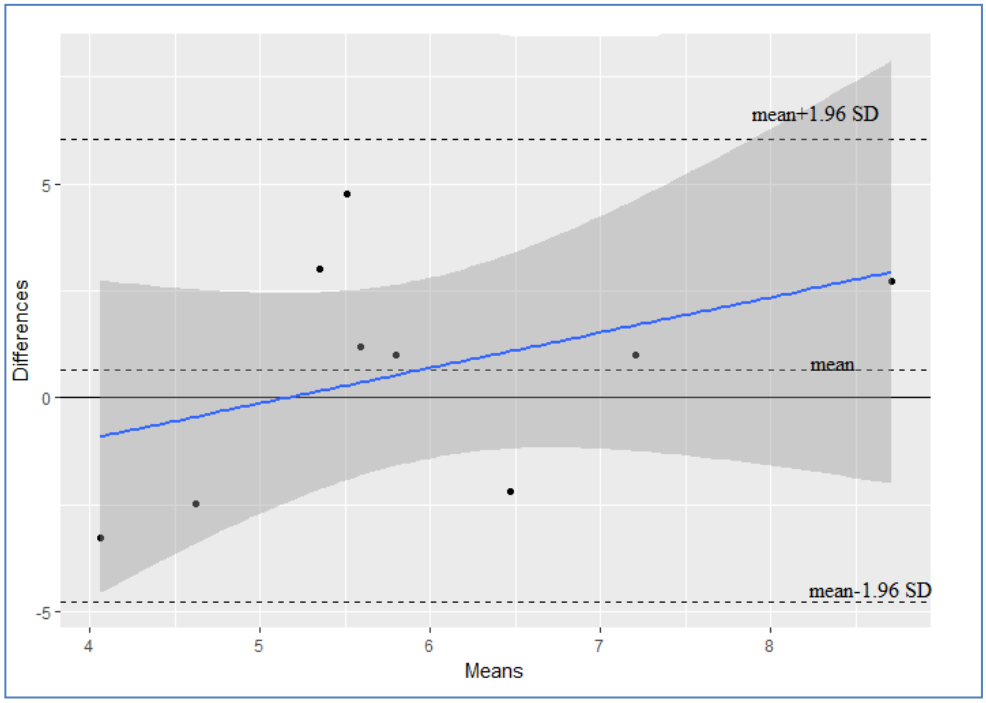


**Figure 1.** The Bland-Altman plot for the agreement between ELISA and LC-MS/MS.

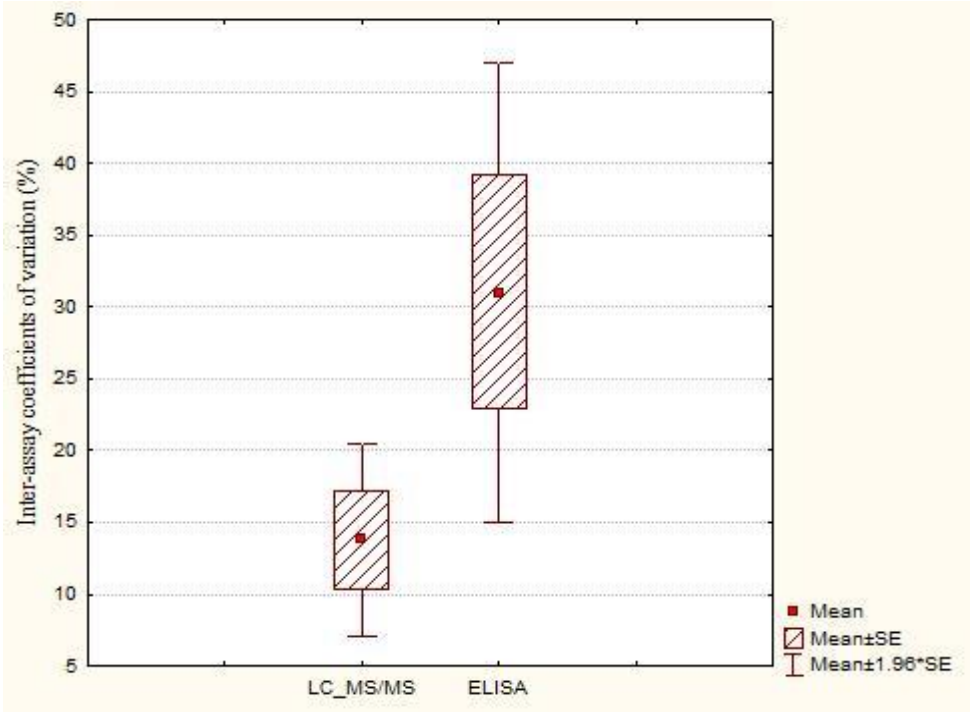


**Figure 2.** The difference between inter-assay coefficients of variation for ELISA and LC-MS/MS.

Earlier studies showed that ELISA displayed less accuracy than LC-MS/MSat lower tacrolimus concentrations (STAATZ & al [14]). This decrease in accuracy has a greater clinical impact while monitoring stable patients after the first year of renal transplant as the tacrolimus trough level target decreases with the time from transplant for all solid organ grafts. A linear correlation (y = 1.0172x + 0.3742, R2 = 0.9630) between the assay results by LC-MS/MS to ELISA in blood has been previously obtained, but the measurement values of ELISA (19 +/- 9)

were significantly higher than that of LC-MS/MS (18 +/- 9, P < 0.01) (LI & al [15]). Our finding regarding the greater inter-assay coefficient of variation for ELISA supports the data by Li et al In a clinical setting, these results might determine unnecessary dose lowering that could potentially be detrimental to long term graft survival, especially in the first 3 months after transplant as already discussed previously.

At tacrolimus concentrations ranging from 0.9 to 29.5 ng/mL, comparing results of a newly developed sandwich ELISA with LC-MS/MS showed a linear regression: sandwich = 0.99 × LC-MS/MS + 0.10 ng/mL, r = 0.991, Sy|x = 1.08 ng/mL (WEI & al [16]). This design appears to be a better solution in blocking metabolite cross-reactivity, which has been noted as the main issue with competitive ELISA kits **.**

The CVs of LC-MS/MS for therapeutic drug monitoring of tacrolimus for the patient samples ranged from 2.0% to 5.4% (ANNESLEY & al [17]) and LC-MRM-MS was commonly used in routine clinical dosage of tacrolimus (CARR & al [18]). All methods that used MS/MS or LC-MS/MS proved more difficult from a practical point of view, requiring specialized staff and thus yielding results lower, albeit with a much better specificity. ELISA methods proved more practical and are more widely available; unfortunately more and more data suggests that competitive ELISA methods significantly overestimate the concentrations of immunosuppressant (TSZYRSZNIC & al [19]). LC-MS/MS has superior specificity which allows it to be less susceptible to interference, but this methodology lacks standardization (MCSHANE & al [20], PATRICHE (LISĂ) & al [21]). Therefore high performance liquid chromatography with various detection methods and immunoassay methods remain the mainstay therapeutic drug monitoring tools (MIKA & STEPNOWSKI [22]). We believe that in the case of calcineurin inhibitors (CNI) in general, and tacrolimus in pediatric renal transplant patients in particular, there is a clear advantage in using LC-MS/MS in guiding immunosuppressive regimens. This being said, we should aim to better define the link between appropriate drug level control and clinical or histological outcomes such as GFR decline, overall renal graft survival and biopsy proven long term CNI toxicity.

## Conclusion

The distribution of tacrolimus concentrations for ELISA and LC-MS/MD did not show significant variations over time and the difference between the measurements is within the limits of the concordance. But the inter-assaycoefficient of variation for the ELISA was shown to have a wider range of variability than for LC-MS/MS, thus ELISA tests seem to be less precise in determination of tacrolimus for renal transplant pediatric patients leading to possible unnecessary dose adjustments or overestimation of treatment efficacy. LC-MS/MS could result in fewer dose changes and an overall better immunosuppression control.

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## Conflict of Interest

No conflict of interest declared.

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