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An HPLC/MS/MS assay for tacrolimus in patient blood samples
Correlation with results of an ELISA assay

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Abstract

An HPLC/MS/MS assay for tacrolimus in whole blood using FR900520 as an internal standard was validated over the standard curve range of 0.100–10.040 ng ml^{-1}. The calibration curve for tacrolimus in human blood gave a slope of 0.2481, an intercept of 0.007, and a correlation coefficient (r) of 0.9996, with no interference noted from human blood, analyte, or internal standard stock solutions. Use of EDTA or heparin as the preservative in blood resulted in no significant differences. Samples were stable for at least the time required to assay the maximum number of samples that could be placed in the automated system. The limit of sensitivity of the assay was set at the concentration of the lowest nonzero standard tested, i.e., 0.100 ng ml^{-1}. However, validation of the assay to a limit of 0.010 ng ml^{-1} is currently underway. The within-run and between-run precision and accuracy of the method were determined for four quality control samples. The highest CV was seen at 0.1 ng ml^{-1} (17.6% within-run and 15.9% between-run), with other CV < 5%. The recovery ranged 79.6–81.3% for tacrolimus over the range 0.3–8.0 ng ml^{-1} and was 63.10 ± 1.37% for FR900520. There was a linear correlation ($r^2 = 0.963$) between assay results by HPLC/MS/MS and ELISA in whole blood from atopic dermatitis patients treated with topical tacrolimus ointment. The difference between the means ± S.D. determined by HPLC/MS/MS (1.22 ± 1.46 ng ml^{-1}) and ELISA (1.12 ± 1.29 ng ml^{-1}) was significant by a paired t-test ($P < 0.001$). Similarly, there was a linear correlation ($r^2 = 0.841$) between assay results by HPLC/MS/MS and IMx in whole blood from solid organ transplant patients treated with tacrolimus. The difference between the means was significantly higher ($P < 0.001$) for the IMx (15.80 ± 8.37 ng ml^{-1}) than the HPLC/MS/MS (13.42 ± 6.87 ng ml^{-1}). 1997 Published by Elsevier Science B.V.

Keywords: Tacrolimus; FK506; HPLC/MS/MS; Immunoassay; Blood

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1. Introduction

FK506 (tacrolimus, Prograf®) is a 23-membered ring, 822 dalton macrolide of actinomycete origin discovered in 1984 in Tsukuba, Japan [1,2]. This novel immunosuppressant agent is approved in the US, Europe, and Japan for prevention of allograft rejection in liver transplant patients and is in clinical trials in kidney transplantation. In addition, orally administered tacrolimus has been used in the treatment of autoimmune disorders at the University of Pittsburgh, including recalcitrant psoriasis and pyoderma gangrenosum [3,4]. A topical tacrolimus formulation (0.3%) currently is in Phase 1/2 trials sponsored by Fujisawa USA, in atopic dermatitis patients.

Therapeutic drug monitoring (TDM) is recommended for monitoring trough blood/plasma tacrolimus levels as an aid in adjusting the dose to prevent rejection and toxicity [5–7]. Two commercially available assays are used to monitor tacrolimus levels, especially in whole blood: Abbott IMx® and PRO-TRAC™ by Incstar [5–7]. These are immunoassays (MEIA and ELISA) which utilize the same monoclonal antibody to tacrolimus. Both of these assays are adequate for therapeutic monitoring in the early posttransplant phase where doses and consequent blood trough levels are higher. Later posttransplant in stable patients [8] and in other indications where lower tacrolimus doses are planned, these assays are limited by the lower limits of quantitation (5 ng ml^{-1} for IMx and 0.5 ng ml^{-1} for PRO-TRAC). In addition, because they are nonspecific, they do not distinguish tacrolimus from metabolites which may increase in concentration later during treatment or during liver dysfunction [8]. For these reasons, more specific assays with better sensitivity are required, especially in patients receiving lower doses of tacrolimus.

HPLC/MS/MS is a sensitive and selective procedure which could prove useful in TDM for tacrolimus if a central laboratory can be established. In this study, an HPLC/MS/MS assay for tacrolimus was validated and the results were compared with those from an ELISA and IMx in patient blood.

2. Materials and methods

Tacrolimus and the internal standard, FR900520, were obtained from Fujisawa Pharmaceutical, Osaka, Japan. These two molecules differ in the absence of C37 in FR900520 and the addition of two hydrogens to the now terminal C36. Methanol, cyclohexane, dichloromethane, ammonium acetate, and ammonium hydroxide were obtained from Aldrich Chemicals (Milwaukee, WI).

Tacrolimus stock solutions containing 100.0 or 10.0 μg ml^{-1} and FR900520 solutions containing 100.0 or 10.0 μg ml^{-1} were made in methanol. An FR900520 internal standard solution for spiking (50 ng ml^{-1}) was made by diluting 1.0 ml of the 10 μg ml^{-1} solution with 199 ml methanol and an internal standard solution (50 ng ml^{-1}) by diluting 1.0 ml of the 10 μg ml^{-1} solution with 199 ml water. A reference solution contained 10 ng tacrolimus and 100 ng FR900520 ml^{-1} and an LOQ reference solution contained 1 ng tacrolimus and 100 ng FR900520 ml^{-1}. All solutions were stored at -22 ± 10°C except the FR900520 internal standard solution (in water) which was stored at 4 ± 6°C and was expected to be stable for 1 month.

Tacrolimus was extracted from whole blood samples (spiked or patient) preserved with EDTA using a solution of dichloromethane:cyclo-hexane (2:3; v/v) prior to HPLC/MS/MS analysis. To 0.5 ml of whole blood in a 16 ± 100 mm screw cap tube was added 100 μl of internal standard solution or water (for blanks), 1.0 ml water and 5 ml of the extraction solution. This was mixed on a reciprocating shaker for 1 h at low speed and this was centrifuged at 3000 rpm for 10 min also at room temperature. The organic layer was transferred to another tube leaving at least 0.5 cm of organic phase and evaporated to dryness. The residue was reconstituted with 50 μl methanol and transferred to an injection microvial. Into the HPLC/MS/MS system, 5 μl of this was injected.

2.1. Immunoassays

The ELISA procedure used in this study is based on the original assay [9] and was previously

reported [10]. Tacrolimus was extracted from whole blood with methanol and processed by the PRO-TRAC™ assay. The IMx procedure used in the current study also was previously reported [11,12].

2.2. HPLC/MS/MS

The HPLC/MS/MS system consisted of a Hewlett Packard 1090 Series II chromatograph with an ODS-Hypersil 10 cm × 2.1 mm, 5 μm analytical column, a PE SCIEX (Perkin-Elmer Sciex) API III MS/MS (atmospheric pressure ionization) detector and an Apple Macintosh Quadra data system. The HPLC elution was isocratic using a mobile phase made by adding 990 ml of methanol to 10 ml of 0.005 M ammonium acetate adjusted to pH 9.0 by adding 28–30% ammonium hydroxide solution. The column pressure was 20–100 barr, the flow rate was 0.3 ml min^{-1}, and the time was 1.5 min.

The TurboIon Spray nebulizer was heated to 400°C and the interface heater to 60°C. The auxiliary flow (N_2) was set at 6.0 l min^{-1}, the nebulizer flow (N_2) at 0.6 l min^{-1} and the curtain gas (N_2) at 1.2 l min^{-1}. The source delta pressure was 1.2 psi and the collision gas (argon) thickness was 300×10^{13} molecules cm^{-2}. The detection mode was multiple reaction monitoring from 0 to 1.5 min with quantitation by the peak-area ratio and regression by 1/concentration linear. The dwell time was 300 ms and the collision energy was 40 V. The m/e for tacrolimus was 802.5–168.2 and that for FR900520 was 790.6–128.0. The retention times were 0.95 min for the internal standard and 0.97 min for tacrolimus.

Nine calibration standards (10.0, 9.0, 7.5, 5.0, 2.5, 1.2, 0.5, 0.2 and 0.1 ng ml^{-1} in blood), a zero, a blank and three quality control (QC) samples (8.0, 4.0 and 3.0 ng ml^{-1}) were run in every series of tacrolimus assays. For the validation procedure, a fourth QC sample of 0.1 ng ml^{-1} was also run. Spiked samples contained tacrolimus over the concentration range 0.100–10.040 ng ml^{-1}.

2.3. Patients

Paediatric and adult (≥ 13 years) atopic dermatitis patients were enrolled in a pharmacokinetic study of tacrolimus (0.3%) ointment. Details of the patients and the results of this study will be reported separately (submitted). Tacrolimus concentrations were determined by HPLC/MS/MS and ELISA in whole blood from adults treated facially over 100 cm^2 and over 1000 cm^2 or 5000 cm^2 of body surface and children aged 3–6 years treated over 50 cm^2. Patients were treated daily for 8 days (once on days 1 and 8 and twice on days 2–7) followed by a 3 day washout period.

Whole blood was obtained from solid organ transplant (liver and kidney) patients receiving primary tacrolimus immunosuppression at the University of Chicago. Samples were obtained from 101 patients and tacrolimus concentrations were determined by HPLC/MS/MS and IMx. Those samples giving values < 5 ng ml^{-1}, the limit of quantitation by IMx, were excluded from the analysis, leaving 83 samples for comparison.

3. Results

The HPLC/MS/MS procedure was validated over the standard curve range of 0.100–10.040 ng ml^{-1}. The calibration curve for tacrolimus in human blood gave a slope of 0.2481, an intercept of 0.007, and a correlation coefficient (r) of 0.9996. Human blood (15 blank pools) and analyte and internal standard stock solutions did not contain any interfering substances. A cross-validation study showed that use of EDTA or heparin as the preservative in blood resulted in no significant differences. Stability data showed stability of samples for 67 days at −22°C, 6.5 h at 20°C, in the autosampler at 20°C for 2.8 h and in methanol at 20°C for 44.42 h. In addition, samples were stable through three freeze-thaw cycles. Stock solutions of tacrolimus in methanol were stable for 127 days and of FR900520 in methanol for 92 days at −22°C.

The limit of sensitivity of the assay was set at the concentration of the lowest nonzero standard, 0.100 ng ml^{-1}. However, validation of the assay

Table 1
Quality control sample between-run and within-run batch precision and accuracy

Parameter	QC sample nominal concentration (ng ml^{-1})			
	0.300	4.004	8.008	0.100
Between run				
Mean ± S.D.	0.308 ± 0.004	3.959 ± 0.11	7.900 ± 0.258	0.095 ± 0.015
CV	1.5%	2.8%	3.3%	15.9%
% Nominal	102.7	98.9	98.7	94.9
Within run				
Mean ± S.D.	0.323 ± 0.013	4.031 ± 0.091	7.963 ± 0.281	0.114 ± 0.020
CV	4.1%	2.3%	3.5%	17.6%
% nominal	107.7	100.7	99.4	113.5

to a limit of 0.010 ng ml^{-1} is currently underway. The within-run and between-run precision and accuracy of the method for four quality control samples are shown in Table 1. The highest CV was seen at 0.1 ng ml^{-1} (17.6% within-run and 15.9% between-run), with other CV < 5%.

The absolute recovery of FK506 in human blood was determined by comparing extracted QC samples at low, medium and high QC concentrations, to unextracted calibration standard solutions representing 100% recovery. The absolute recovery of FR900520 (IS) in human blood was determined by comparing replicate IS samples at 2.52 ng ml^{-1} to a seven-point unextracted IS calibration curve representing 100% recovery (FK506 was used as an external standard). The recovery ranged 79.6–81.3% for tacrolimus (Table 2) and was 63.10 ± 1.37% for FR900520 (CV = 2.2%).

The correlation between assay results by HPLC/MS/MS and ELISA in whole blood from atopic dermatitis patients receiving topical tacrolimus showed higher values determined by HPLC/MS/MS ($y = 1.08x + 0.01$, $N = 306$), an intercept close to zero, and a correlation coefficient (r^2) of 0.963. The difference between the means determined by HPLC/MS/MS (1.22 ± 1.46 ng ml^{-1}) and ELISA (1.11 ± 1.29 ng ml^{-1}) was significant by a paired t-test ($P < 0.001$). The correlation between HPLC/MS/MS and ELISA results for individual atopic dermatitis patients over time is shown in Figs. 1 and 2. The ELISA results are the average of duplicate assay, while the LC/MS/MS results are a single determination.

The correlation between assay results by HPLC/MS/MS and IMx in whole blood from solid organ transplant patients treated with tacrolimus gave an equation of $y = 0.690x + 2.506$ ($N = 83$), an intercept of about 2 ng ml^{-1}, and a correlation coefficient (r^2) of 0.841. The difference between the means was significantly higher ($P <$ 0.001) for the IMx (15.80 ± 8.37 ng ml^{-1}) than the HPLC/MS/MS (13.42 ± 6.87 ng ml^{-1}).

Table 2
Percent recovery of tacrolimus in quality control samples

Parameter	0.300 ng ml^{-1}	4.004 ng ml^{-1}	8.008 ng ml^{-1}
Mean ± S.D. (%)	79.61 ± 5.865	81.13 ± 1.316	81.33 ± 1.840
CV%	7.4	1.6	2.3
N	10	10	10

4. Discussion

The current HPLC/MS/MS procedure was a reliable and sensitive assay for tacrolimus in whole blood, with a limit of sensitivity of 0.10 ng ml^{-1}. This makes the limit of sensitivity 50-fold lower than that of the currently available IMx (5 ng ml^{-1}) and five-fold lower than that of the ELISA (0.5 ng ml^{-1}) in whole blood. While improvements in the IMx may lower the sensitiv-

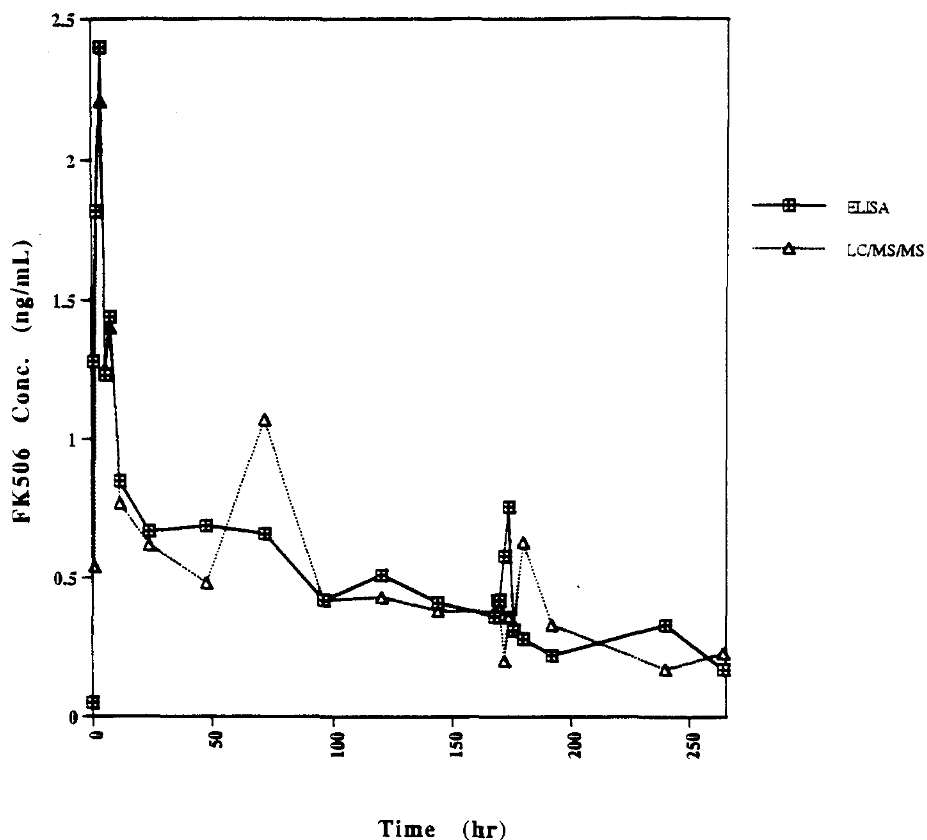

Fig. 1. Correlation between ELISA and HPLC/MS/MS for adult patient received 15.0 g 5000 cm^{-2} of 0.3% tacrolimus ointment

ity to 3 or even 1 ng ml^{-1}, these are still manyfold higher than the current HPLC/MS/MS assay. Similarly, improvements in the ELISA may lower the sensitivity to near that of the current HPLC/MS/MS assay, but these will be higher than the expected limit of 0.010 ng ml^{-1} for the latter assay.

The CV for the current assay was highest (16–18%) at the limit of quantitation, but $< 5\%$ at higher concentrations. This compares favorably with CV of up to 20% found acceptable for enzyme immunoassays. The recovery of tacrolimus was stable at about 80% over the tested range of 0.3–8.0 ng ml^{-1}. This is lower than the recovery reported for extraction procedures used for immunoassays, which are generally 85–95%. Variable recovery was previously reported for an HPLC/MS procedure with recovery of 78.9–90.4% over the range of 1–25 ng ml^{-1} [13].

A good correlation was found between the results determined by HPLC/MS/MS and ELISA or IMx in patient samples in the current study. Good correlations have been previously reported between HPLC/MS and IMx tacrolimus assays [13], but not between HPLC/MS and ELISA tacrolimus assays [14].

In atopic dermatitis patients, a comparison was made between the ELISA and HPLC/MS/MS assays following topical treatment with tacrolimus ointment. The IMx could not be used in these patients as the mean blood trough concentrations were well below the limit of quantitation. Interestingly, a 8.5% higher concentration of tacrolimus was measured by HPLC/MS/MS than by ELISA ($P < 0.001$). The reason for this is unclear, but is unlikely to be related to metabolites.

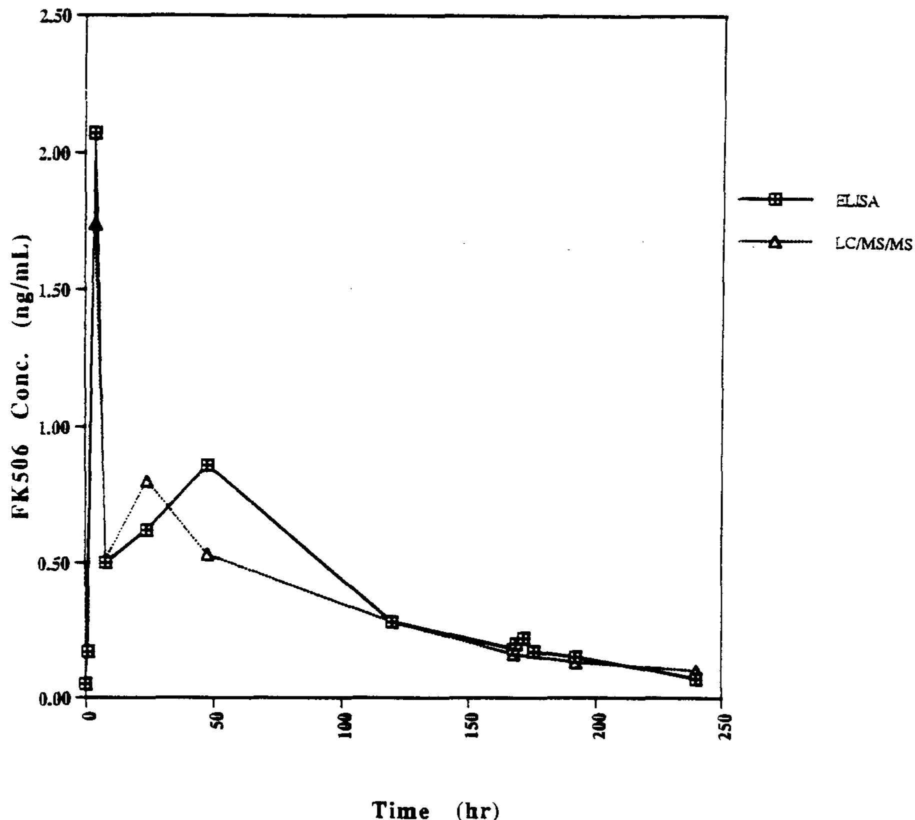

Fig. 2. Correlation between ELISA and HPLC/MS/MS for pediatric patient received 0.25 g 50 cm^{-2} of 0.3% tacrolimus ointment

In solid organ recipients, there was a good correlation between tacrolimus concentrations in whole blood determined by HPLC/MS/MS and IMx, although the latter assay gave 15.1% higher ($P < 0.001$) results. This could be due to measurement of metabolites by the IMx.

TDM of tacrolimus has been recommended as an aid to dosing and whole blood is the recommended matrix. TDM is most often used to minimize toxicity, but may also be useful in guiding dosing. No correlation has been shown between blood or plasma tacrolimus trough levels and efficacy, however, and only some correlation between nephrotoxicity and trough levels [15,16]. The extent to which this is due to the nonspecificity of the assays is unknown, although most metabolites have only a fraction of the immunosuppressant activity of tacrolimus while some interact considerably with the antibody used in immunoassays.

HPLC/MS/MS may prove useful in TDM of tacrolimus, as the assay is reliable, sensitive, and comparable in cost to IMx. The good correlation of the current HPLC/MS/MS assay with previous immunoassays is comforting, as clinical impressions of tacrolimus therapeutic windows, drawn using previous assays, may continue. However, the increased specificity of the HPLC/MS/MS may allow correlations previously not seen to become evident. In addition, the increased sensitivity of the HPLC/MS/MS assay will allow accurate determinations of tacrolimus concentrations in stable organ transplant patients and other patients treated with lower doses.

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