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Assay of FK 506 in Plasma

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FK 506 is a potent immunosuppressive agent in vitro and in vivo in animals and, as discussed extensively in this symposium, in humans. In contrast to the early use of CyA, an assay for the measurement of FK 506 has been available for use in these early studies.[1] We present our experience with the modified enzyme immunoassay (EIA) procedure we have used in preclinical studies and in the early clinical trials of FK 506.

MATERIALS AND METHODS

FK 506 Assay

The FK 506 assay is an EIA using a mouse monoclonal anti-FK 506 antibody (Fujisawa Pharmaceuticals Co Ltd, Osaka, Japan). Briefly, anti-mouse IgG is adsorbed onto a 96-well flat-bottomed microtiter plate (Flow Labs, McLean, VA) overnight at 4°C and the plate is blocked with 0.5% albumin. FK 506 (Fujisawa) standards (a zero standard and from 0.1 to 5.0 ng/ml) are prepared 10× concentrated in methanol (MeOH). One hundred microliters of plasma is pretreated with 1 ml of 0.1 N HCl, and 100 μl of FK 506-free plasma is used for the standards. Ten microliters of standard solution or MeOH blank is then added to each sample. One milliliter of the treated sample is loaded onto a C-18 mini-column (Sep-pak, Waters, Milford, MA) that has been prewet with MeOH and acetic acid. The samples are washed with acetic acid and eluted with 3.0 ml of MeOH. The eluate is evaporated to dryness and reconstituted with peroxidase labeled FK 506 (Fujisawa). Reconstituted sample and monoclonal antibody are placed in each well, and competitive binding occurs overnight at 4°C with gentle agitation. Unbound FK 506 is removed, and the activity of the bound FK 506-peroxidase conjugate is measured by the increase in optical density (OD) at 492 nm after a 20-minute incubation with o-phenylenediamine (Sigma, St Louis, MO) substrate.

The standard curve is linearized using a log-logit transformation, and controls and unknowns are calculated from the regression line.

Precision, Accuracy, and Recovery Studies

Pools of human plasma were spiked with FK 506 to concentrations of 0.5, 1.0, and 2.0 ng/ml. Aliquots of these samples were taken with each run to determine accuracy and precision. Recovery of the extraction procedure was assessed by diluting 10 μl of the standards to 3 ml in MeOH and processing them further as mini-column eluates.

Interference

Samples with a variety of bilirubin and FK 506 concentrations were prepared using normal plasma and plasma from a jaundiced patient not on FK 506; the plasma was spiked with FK 506. A similar matrix was created with plasma contaminated with hemolyzed red blood cells. FK 506 was measured in samples sent for CyA determination to look for cross-reactivity.

Separation of Plasma

Whole blood samples from patients on FK 506 are mixed thoroughly and divided into two aliquots. One is left at room temperature and centrifuged, the second is incubated at 37°C for a minimum of 30 minutes, then spun at 37°C. Plasma from both aliquots is stored at 4°C until assayed.

RESULTS

FK 506 Enzyme Immunoassay

Measurements of OD vary considerably from day to day, with the zero standard ranging from 0.8 to 2.0. However, the variation is consistent within each run. The coefficient of variation (CV) of the FK 506 assay is 27% at 0.5 ng/ml, 23% at 1.0 ng/ml, and 15% at 2.0 ng/ml. The assayed values of these samples are 0.47, 0.93, and 2.1 ng/ml, respectively, with accuracies thus ranging from 93% to 95%. The recovery of the extraction ranges from 100% at 0.5 ng/ml to 125% at 5.0 ng/ml. The linear range is 0.1 to 5.0 ng/ml. Samples with higher concentration are diluted with FK 506-free plasma.

Interference Studies

Hemolysis does not interfere with the FK 506 assay, even when marked hemolysis is present. A low FK 506 concentration sample measured 0.1, 0.1, and < 0.1 ng/ml when slight, moderate, or marked hemolysis was present. Another measured 0.5, 0.3, and 0.3, respectively, and a third measured 1.1, 1.0, and 1.2. Similarly, at bilirubin concentrations of 10, 160, and 310 μmol/L (0.5, 9, and 18 mg/dl), FK 506 measured < 0.1, < 0.1, < 0.1, respectively, in one sample, 0.5, 0.4, and 0.4 in a second, and 1.7, 1.7, and 1.9 in a third. CyA concentrations of up to 2,000 ng/ml by FPIA (TDx^R) did not cross-react in the FK 506 assay, reading < 0.1 ng/ml FK 506.

Plasma Separation

For 220 samples from eight patients, separation of plasma at both room temperature and at 37°C was done. Plasma separated at room temperature contained <75% of the

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amount of FK 506 compared with its 37°C aliquot (room temperature = 0.75 × [37°C] − 0.2; $r = 0.9763$).

Dose Response

In 30 patients, the relationship between dose of FK 506 and 12-hour trough concentrations was studied with a correlation coefficient of $r < 0.3$. To eliminate the interpatient variability, one patient was identified in whom an intrapatient dose-response curve could be examined. Regression revealed a correlation coefficient of $r < 0.33$ in this patient.

DISCUSSION

The FK 506 assay has performed satisfactorily in our laboratory. Measurement of FK 506 concentrations in animal studies has shown wide interspecies variation in kinetics[2] and dosing requirements,[3] and in vitro studies have shown interspecies variations in sensitivity of lymphocytes to FK 506.[4] It was therefore felt that the assay system would be necessary for adjusting dosing and determining pharmacokinetics in phase I clinical trials.

Although the OD varies from day to day, we were unable to discern either a consistent pattern or the reason for this variation. Despite this effect, the shape of the standard curve is uniform each day, and results of the control samples are consistent. The 15-27% CV compares reasonably with that of CyA—our current CyA high-pressure liquid chromatography assay has a CV of 14-22% and our radioimmunoassay has a CV of 10-19%. Automation of CyA for the TDx^R has improved the CV to 7-14%. Two major factors accounting for the lesser precision of the FK 506 assay are the low concentrations of the agent and the inexactness of an EIA. The calculated accuracy and recoveries are not significantly different from 100%.

Since FK 506 kinetics differ in patients with liver failure,[5] it was essential to demonstrate that this is not due to bilirubin interfering with the assay. Similarly, FK 506 measurements on hemolyzed samples are accurate.

Initial pharmacokinetic studies in humans and experience with CyA have suggested that the distribution of FK 506 between red blood cells and plasma may be temperature-dependent. Indeed, we did find such a temperature dependence and have switched to 37°C separation to reflect in vivo conditions. This has allowed for reliable studies on the distribution and elimination of FK 506.[6]

The poor correlation between dose and plasma concentration of FK 506 is similar to that seen in baboons.[3] However, the safety and efficacy profile seen in the phase I clinical trials may obviate the need for precise and frequent measurement of FK 506 to fine-tune plasma concentrations.[7-9]

The current FK 506 assay has been essential to the preclinical and early clinical trials of FK 506, and measurement of FK 506 will be necessary as clinical trials continue. Measurement of FK 506 concentrations will be necessary early in therapy, especially for patients with liver failure, but routine "FK 506 levels" may not be needed as frequently as they are for CyA.

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