Comparison Between a Liquid Chromatography-Tandem Mass Spectrometry Assay and a Fluorescent Polarization Immunoassay to Measure Whole Blood Everolimus Concentration in Heart and Renal Transplantations

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Various methods [fluorescent polarization immunoassay (FPIA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay] are used for therapeutic drug monitoring of everolimus. The aim of this study is to compare these assays in renal and heart transplantation. The correlation between results was investigated by linear regression in 44 patients (24 heart recipients and 20 renal recipients—137 samples). The comparison between assays was performed by a paired *t*-test. A highly significant correlation was found between FPIA and LC-MS/MS in heart and renal

recipients [$FPIA = 0.851 \times LC-MS/MS + 1.773r^2 = 0.8738$ ($P < 0.001$)]. Paired *t*-tests did not show a significant difference between everolimus whole blood concentrations in the populations of heart and renal recipients or heart recipients or renal recipients. FPIA and LC-MS/MS assays gave consistent overall results although some significant differences were observed in some samples between these methods indicating that FPIA assay has limitations that deserve further investigations. *J. Clin. Lab. Anal.* 22:282–285, 2008. © 2008 Wiley-Liss, Inc.

Key words: everolimus; chromatography; therapeutic drug monitoring; immunoassay

INTRODUCTION

Everolimus is a potent immunosuppressive drug recently approved by the Food and Drugs Administration and the European Medicines Agency to be used in renal and heart transplantation (1). The relationships between clinical efficacy or toxicity and everolimus whole blood concentration and the high interindividual variability of these concentrations were established suggesting the usefulness of therapeutic drug monitoring of everolimus (2). Consequently, various chromatographic assays, which are reference methods, and a fluorescent polarization immunoassay (FPIA) were developed to measure everolimus whole blood concentration. Previous works compared the FPIA with high-performance liquid chromatography assays coupled

with UV detection in renal and/or heart transplantations (3,4) or with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay in renal transplantation (5). No study investigated this relationship between FPIA and LC-MS/MS simultaneously in heart and renal transplantations. Our aims are to compare results of everolimus whole blood concentrations between both these methods in heart and renal recipients

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and to compare the relationships between FPIA and LC-MS/MS in heart and renal recipients.

MATERIALS AND METHODS

Patients

The cohort is composed of 44 patients (24 heart recipients and 20 renal recipients). Mean age of the patients was 55.04 ± 14.06 years (11 females, 33 males). The immunosuppressive drugs associated with everolimus are cyclosporine, prednisone, tacrolimus, and mycophenolic acid in various drugs regimens. A total of 137 trough blood samples were collected. The mean number of samples per patient was 2.57 (range 1–9). Informed consent was not required for the patients as the data were derived from an ongoing drug monitoring program and were reported in accordance with ethical guidelines.

LC-MS/MS Assay

The determination of everolimus whole blood concentration was adapted from a previously validated assay (6). Samples were prepared by adding 200 μ l of a diluting solution [methanol/0.2 M ZnSO_4 (80/20 v/v)] of demethoxyrapamycin (50 ng/ml), which is used as the internal standard, to 100 μ l of either blood, calibrators, or controls (calibrators and controls were obtained from Chromsystems, Munich, Germany) and vortex mixing. After centrifugation at 13,000g for 15 min, 150 μ l of supernatant was transferred to an autosampler vial and 15 μ l was injected in the chromatographic system. This system consists of Agilent (Palo Alto, USA) 1200 Series components including binary pump, isocratic pump, column oven, and autosampler. The hardware configuration included an Applied Biosystems/MDS Sciex (Toronto, Canada) API 3200 equipped with a turboion-spray source. Two dimension chromatography was performed. First dimension chromatography was accomplished via a Applied Biosystems Poros R1:20 2.1×30 mm perfusion column. The binary pump supplied eluent (methanol/water 10/90 (v/v)—2.7 ml/min for 1 min) for enrichment of everolimus and internal standard on Poros column. A back flush elution of everolimus and internal standard is performed by a second eluent (methanol/water 97/3 (v/v), 10 mM $\text{CH}_3\text{COONH}_4$, 0.1 % acetic acid) at a flow rate of 500 μ l/min supplied by the isocratic pump and a second dimension chromatography was performed using a Phenomenex (Torrance, CA) Luna 5 μ m Phenyl Hexyl, 2×50 mm column housed in oven at 60°C. Mass spectrometric detection was performed in positive ion mode using selected reactant monitoring (everolimus m/z 975.5 $\rightarrow$ 908.4, internal standard m/z 901.5 $\rightarrow$ 816.4). A

seven-point calibration curve (0, 2, 6, 12, 18, 26, 46 ng/ml) was constructed using a quadratic regression ($1/x$ weighting). Quality controls (3.04, 5.06, 10.4, 35.6 ng/ml) were analyzed in each batch. The method was found to be accurate (inaccuracy <10%) and showed good precision (imprecision <10%). The limit of quantitation is 0.5 ng/ml.

Fluorescent Polarization Immunoassay

The assay is performed according to manufacturers' instructions (Seradyn, Indianapolis, USA). Calibrators, controls, or patient blood samples (300 μ l) were mixed with methanol (350 μ l) and precipitation reagent (50 μ l). After centrifugation, the supernatant (400 μ l) is transferred into reaction cells and submitted to a TDxFLx instrument (Abbott Diagnostics, Abbott Park, IL) for automated analysis. The calibrators and quality controls (low concentration range: 2.20–5.11 ng/ml; medium concentration range: 6.95–11.66 ng/ml; high concentration range: 13.25–21.21 ng/ml) were provided with the kit. The calibrators were analyzed in duplicate and three levels of quality controls were added during each batch of analysis. The limit of quantitation is 2 ng/ml.

Statistical Analysis

The correlations between FPIA and LC-MS/MS measurements of everolimus whole blood concentrations were investigated by linear regression ($P < 0.05$ for significance). The comparison between everolimus whole blood concentrations measured by FPIA and LC-MS/MS was performed by a paired t -test ($P < 0.05$ for significance).

RESULTS

A highly significant correlation was found between FPIA and LC-MS/MS in heart and renal recipients

Fig. 1. Correlation between FPIA and LC-MS/MS measurements of everolimus whole blood concentrations in heart and renal recipients (137 samples, 44 patients). $\text{FPIA} = 0.851 \times \text{LC-MS/MS} + 1.773$ ($r^2 = 0.8738$ ($P < 0.001$)). FPIA, fluorescent polarization immunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

Fig. 2. Correlation between FPIA and LC-MS/MS measurements of everolimus whole blood concentrations in heart recipients (71 samples, 24 patients). $FPIA = 0.8291 \times LC-MS/MS + 1.9669$ ($r^2 = 0.7979$ ($P < 0.001$)). FPIA, fluorescent polarization immunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

Fig. 3. Correlation between FPIA and LC-MS/MS measurements of everolimus whole blood concentrations in renal recipients (66 samples, 20 patients). $FPIA = 0.8587 \times LC-MS/MS + 1.6723$ ($r^2 = 0.9071$ ($P < 0.001$)). FPIA, fluorescent polarization immunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

[$FPIA = 0.851 \times LC-MS/MS + 1.773$ ($r^2 = 0.8738$ ($P < 0.001$))] (Fig. 1). This result is consistent with the results of an analysis taking into account the transplanted organ: $FPIA = 0.8291 \times LC-MS/MS + 1.9669$ ($r^2 = 0.7979$ — $P < 0.001$) (Fig. 2) in heart recipients and $FPIA = 0.8587 \times LC-MS/MS + 1.6723$ ($r^2 = 0.9071$ — $P < 0.001$) (Fig. 3) in renal recipients. Paired *t*-tests did not show a significant difference between everolimus whole blood concentrations in the populations of heart and renal recipients or heart recipients or renal recipients.

DISCUSSION

A highly significant correlation and consistent results were found between everolimus concentration measured by FPIA and LC-MS/MS. Moreover, no influence of the transplanted organ (heart or kidney) on this

relationship between FPIA and LC-MS/MS was shown. However, important differences between both methods, which could lead to incorrect therapeutic decisions, were observed in some individual samples according to previous studies (3,4,5). These results moderate the performance level of FPIA assay although these differences concern a limited number of samples. The overestimation of FPIA results is usually explained by the crossreactivities of everolimus metabolites. However, these crossreactivities of the major metabolites of everolimus cannot fully explain this bias (7). A complementary explanation of this overestimation proposed by Salm et al. (5) was calibration differences between methods as previous studies used calibrators prepared in-house. No systematic overestimation of FPIA results was found in our work contrary to previous studies. Our LC-MS/MS results are based on calibrations performed with calibrators commercially available suggesting that this calibration could attenuate this overestimation. Moreover, the variability of LC-MS/MS results is affected by the problem of matrix effect, which arises in LC-MS/MS assays especially with limited sample clean-up. According to this matrix effect caused by interpatient matrix variability, the internal standard selection for the LC-MS/MS assay of everolimus in blood can modify ionization efficiency during the transfer of everolimus from the liquid phase to the gas phase producing significant differences in the estimation of the everolimus concentrations between LC-MS/MS assays (8). Large differences were found between everolimus concentrations measured by LC-MS/MS assays based on different internal standards, whereas results were consistent between LC-MS/MS assays based on a similar internal standard (9). This phenomenon could also participate in the explanation that no systematic overestimation of FPIA result was found contrary to previous studies based on chromatography with UV detection (3,4) or LC-MS/MS assay with another internal standard (5).

In conclusion, FPIA and LC-MS/MS assays provided consistent overall results although some significant differences were observed between these methods in some samples indicating that FPIA assay has limitations that deserve further investigations.

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