Rapid Estimation of Whole Blood Everolimus Concentrations Using Architect Sirolimus Immunoassay and Mathematical Equations: Comparison With Everolimus Values Determined by Liquid Chromatography/Mass Spectrometry

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United States Food and Drug Administration (FDA) in 2010 approved the use of immunosuppressant drug everolimus, which requires therapeutic drug monitoring in whole blood. Taking advantage of structural similarity between sirolimus and everolimus we attempted to rapidly estimate everolimus concentration from apparent sirolimus concentration obtained by using Architect sirolimus immunoassay and mathematical equations (both polynomial and linear). Mathematical equations were derived by curve-fitting methods based on observed apparent sirolimus concentration and true everolimus concentration determined by a liquid chromatography combined with mass spectrometry (LC/MS)

method using eight everolimus standards (concentration range 1–30 ng/mL) prepared in whole blood. In order to determine the validity of our approach, we analyzed 12 specimens from patients receiving everolimus using both Architect sirolimus assay and LC/MS method. We observed good correlation between calculated everolimus values and true everolimus values as determined by LC/MS. However, if a patient is switched from sirolimus to everolimus, then sirolimus immunoassay can roughly estimate everolimus concentration plus any residual sirolimus present in whole blood and it is not possible to calculate everolimus concentration. *J. Clin. Lab. Anal.* 25:207–211, 2011. © 2011 Wiley-Liss, Inc.

Key words: everolimus; sirolimus; architect sirolimus assay; mathematical equation; liquid chromatography; mass spectrometry

Everolimus (Certican[®]; Novartis Pharmaceuticals, Basel, Switzerland) is a proliferation signal inhibitor in the mammalian target of rapamycin drug class, which has recently received FDA (Food and Drug Administration of the United States) approval for therapeutic use as a prophylactic agent to avoid rejection after kidney transplantation. In addition, this drug is also approved for the treatment of renal cell carcinoma and their use as a drug-eluting stent. In clinical trials, everolimus demonstrated immunosuppressive properties in preventing acute rejection in cardiac, liver, lung, and renal transplant recipients (1). Everolimus is a macrolide drug derived by structural modification of sirolimus, a natural product obtained from strains of *Streptomyces hygroscopicus*. Therefore, everolimus is structurally very similar to sirolimus but contains a substituted 2-hydroxyethyl chain at position 40

on the sirolimus molecule (2,3). Chemical structures of sirolimus and everolimus are given in Figure 1.

Everolimus exerts its immunosuppressant activity by blocking the proliferative signals of growth factors thereby preventing cells from entering the S-phase (4,5). Therefore, the mechanism of immunosuppressant by everolimus is broader than calcineurin inhibitors (cyclosporine and tacrolimus), which act by inhibiting T-cell

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Fig. 1. Chemical structures of sirolimus and everolimus.

growth factors such as IL-2. Because of this complementary mechanism of action, everolimus can be used along with calcineurin inhibitors, potentially allowing lower dosages of the latter thus minimizing well-known concentration-related side effects of calcineurin inhibitors (6,7). Everolimus has a much shorter half-life than sirolimus thus allowing twice daily dosing compared with once daily dosing of sirolimus. Hence, steady state can be achieved more quickly within 7 days compared with 13 days required for sirolimus (8). However, similar to other immunosuppressants, therapeutic drug monitoring of everolimus is essential. The recommended therapeutic dosage of everolimus is 3–8 ng/mL of whole blood trough concentration. Toxicity may be encountered at a concentration of more than 8 ng/mL (9).

Although immunoassays are readily available for monitoring cyclosporine, tacrolimus, and sirolimus, currently there is only one FDA approved immunoassay in the market for everolimus in the US market.

According to a communication by Novartis (August 3, 2010), only three reference laboratories in the nation offer therapeutic drug monitoring of everolimus using liquid chromatography combined with mass spectrometry (LC/MS). Unfortunately, LC/MS methods are cumbersome requiring extensive sample preparation and analysis of one specimen at a time in contrast to automated analyzers where specimens can be analyzed in a batch. In addition, LC/MS equipment is expensive and many hospital laboratories including some University-based hospital laboratories do not have this technology available. We have recently developed and validated a LC/MS assay for everolimus and we also have an Architect analyzer in our laboratory. We hypothesized that taking advantage of structural similarity between everolimus and sirolimus, it is possible to use Architect sirolimus assay and mathematical equation for rapidly predicting everolimus concentration in whole blood. This approach will be useful for many hospital laboratories without LC/MS facility for rapid estimation of everolimus concentration using sirolimus immunoassay. In this study, we present our findings.

MATERIALS AND METHODS

Pure sirolimus and everolimus standards were purchased from Sigma Chemical Company (St. Louis, MO). The immunoassay kits for sirolimus was obtained from Abbott Laboratories (Abbott Park, IL) and the assay was performed using Architect analyzer (Architect i1000) also available from the Abbott Laboratories. The whole blood Architect sirolimus immunoassay is based on CMIA (chemiluminescent microparticle immunoassay) technology and requires a sample pretreatment using a precipitation reagent (also supplied by the manufacturer) followed by heating and centrifugation. Then the clear supernatant is analyzed using an Architect i1000 analyzer. The calibration range of the assay is 0.0–30.0 ng/mL of sirolimus whole blood concentration with a sensitivity of ≤ 1 ng/mL although the assay was designed to have a functional sensitivity of 2 ng/mL and the assay is linear up to a whole blood sirolimus concentration of 30.0 ng/mL.

We used eight different everolimus standards (concentrations ranging from 1 to 30 ng/mL) for preparing the calibration curve and deriving the mathematical equations. Then apparent sirolimus concentrations were determined using the Architect sirolimus assay and true everolimus concentrations were determined by using the LC/MS method. We also analyzed 12 whole blood specimens collected from patients receiving everolimus using both sirolimus immunoassay and LC/MS. These specimens are kind gift from Thermo Fischer Scientific Diagnostics Division (Fremont, CA). In addition, we

also prepared two whole blood sirolimus pools by combining several specimens from patients taking sirolimus. Then aliquots of these samples, which were further supplemented by various amounts of everolimus and sirolimus concentrations, were measured again using the sirolimus immunoassay. We used only discarded blood for preparing sirolimus pool. These patients' specimens are routinely submitted to our clinical laboratory for therapeutic drug monitoring of sirolimus. Then after reporting the result to the ordering physician and preserving the specimen for 1 month, these specimens are discarded. In order to investigate the cross-reactivity of cyclosporine and tacrolimus with the sirolimus immunoassay, we analyzed the highest whole blood cyclosporine (1,200 ng/mL) and tacrolimus calibrator (50 ng/mL) using the sirolimus immunoassay.

The LC/MS instrument was purchased from Waters Corporation (Milford, MA). For sample preparation, whole blood containing everolimus was treated with 10% methanolic solution containing 0.1 M ammonium bicarbonate. Protein precipitation was achieved using 0.1 M zinc sulfate. Then everolimus along with internal standard (ascomycin) was extracted using an organic extraction solvent (acetone/methanol). Liquid chromatography was accomplished by using a reverse phase C-18 column (Waters Corporation) and quantification was achieved by using m/z 980.5 for everolimus and m/z 814.5 for the internal standard. The assay was linear from 2 to 50 ng/mL everolimus concentration.

RESULTS

Everolimus exhibited significant cross-reactivity with sirolimus immunoassay. When everolimus standards were analyzed using sirolimus immunoassay, the observed cross-reactivity varied from 74 to 100%. Highest cross-reactivity was observed with the standard containing lowest amount of everolimus (Table 1). We calculated cross-reactivity of everolimus with sirolimus immunoassay by dividing apparent sirolimus concentration with true everolimus concentration as obtained by the LC/MS assay. We attempted various method of curve-fitting (linear, polynomial, exponential, logarithmic, and power) to obtain best correction between apparent sirolimus concentration and true everolimus concentration determined by the LC/MS method. For this purpose, we plotted apparent sirolimus concentration data in the "x-axis" and true everolimus concentration in the "y-axis." The rationale for this approach is that when we calculate everolimus concentration from known apparent sirolimus concentration, the "x" value must be known for calculation of the "y" which in this case is the calculated everolimus concentration. In the case of a linear equation ($y = mx + c$, where m is the

TABLE 1. Cross-Reactivity of Everolimus With Sirolimus Assay

Target	Everolimus (ng/mL) measured by LC/MS	Apparent Sirolimus (ng/mL) measured by Architect sirolimus assay	Cross-reactivity (%)
1.0	1.1	1.1	100
2.5	2.4	2.1	88
5.0	5.4	4.3	80
10.0	10.8	8.0	74
15.0	15.3	11.5	75
20.0	20.8	16.4	79
25.0	25.2	19.3	77
30.0	32.1	25.1	78

LC/MS, liquid chromatography combined with mass spectrometry.

Fig. 2. Curve-fitting between sirolimus values obtained by Architect immunoassay and true everolimus concentrations as determined by liquid chromatography/mass spectrometry (LC/MS) using polynomial equation.

slope and c is the intercept), choice of x and y does not matter because this equation can be easily manipulated, but for polynomial fit with an equation; $y = ax^2 + bx + c$, such manipulation is difficult and it is better to calculate the "y" from the known value of the "x" which in this case apparent sirolimus concentration as determined by the sirolimus immunoassay.

We observed good curve-fitting using a polynomial equation where x is the observed apparent sirolimus concentration and y is the true everolimus concentration determined by the LC/MS method:

$$y = -0.0042x^2 + 1.3985x - 0.4322 \quad (r = 0.99)$$

(Fig. 2).

However, we also observed good curve-fitting using the linear model:

$$y = 1.2944x - 0.0689 \quad (r = 0.99)$$

(Fig. 3).

Fig. 3. Curve-fitting between sirolimus values obtained by Architect immunoassay and true everolimus concentrations as determined by liquid chromatography/mass spectrometry (LC/MS) using linear equation.

TABLE 2. Comparison of Calculated Everolimus Concentration Obtained by Using Mathematical Equations With Everolimus Values Determined by Liquid Chromatography/Mass Spectrometry (LC/MS)

Specimen #	Apparent Sirolimus (ng/mL)	Calculated Everolimus (ng/mL)		Everolimus (LC/MS)
		Polynomial	Linear	
1	2.6	3.2	3.3	2.9
2	4.6	5.9	5.9	5.7
3	6.3	8.2	8.1	8.6
4	3.7	4.7	4.7	5.0
5	8.1	10.6	10.4	8.6
6	2.0	2.3	2.5	2.0
7	5.7	7.4	7.3	7.5
8	12.9	16.9	16.6	14.5
9	19.0	24.6	24.4	22.1
10	7.3	9.6	9.3	10.5
11	16.6	21.6	21.3	18.8
12	12.9	16.9	16.6	17.5

Both equations can be used to calculate everolimus concentration based on apparent sirolimus concentration as determined by the Architect sirolimus immunoassay. In Table 2, observed apparent sirolimus concentrations, calculated everolimus concentrations and true everolimus concentrations as determined by the LC/MS method are listed. Both equations produced comparable calculated everolimus concentrations, which matched well with true everolimus concentrations as determined in 12 patients studied. When we plotted calculated everolimus values obtained by using polynomial equation and apparent sirolimus concentrations in the y -axis and true everolimus values as determined by the LC/MS method in the x -axis (reference method), we observed the following regression equations:

$$y = 1.11 \times -0.47 \quad (r = 0.99)$$

Fig. 4. Linear regression analysis showing correlation between everolimus values obtained by using linear equation and apparent sirolimus concentration and everolimus values determined by liquid chromatography/mass spectrometry (LC/MS).

Again when we plotted calculated everolimus values using linear equation and apparent sirolimus concentrations in the y -axis and true everolimus values as determined by the LC/MS method in the x -axis, we observed the following regression equations:

$$y = 1.09 \times -0.38 \quad (r = 0.98)$$

(Fig. 4).

These regression analyses indicated that there was an average 11% positive bias between calculated everolimus concentration obtained by using polynomial equation and the true everolimus determined by the LC/MS method. In addition, when everolimus concentrations were calculated using the linear equation, the average positive bias was 9%. Again both approaches demonstrated excellent correlation coefficients.

The highest bias between calculated everolimus concentration and observed everolimus concentration was positive bias of 23.2% in specimen number 5 using polynomial equation and in patient number 6 (25% positive bias) using linear equation. We observed more than 20% difference between calculated everolimus value and observed everolimus value in only one specimen (specimen number 5) using the polynomial equation and in two specimens (specimen number 5 and 6) using linear equation. Six specimens showed bias less than 10% indicating that there is a good correlation between calculated everolimus concentration and true everolimus concentration as determined by the LC/MS method.

However, if a patient is switched from sirolimus to everolimus this approach is unsuitable for the calculation of everolimus concentration if the residual sirolimus is still in the circulation due to its long half-life. When we further supplemented two whole blood sirolimus pools with various concentrations of everolimus, we observed a rough estimate of a combined concentration

TABLE 3. Effect of Adding Various Amounts of Everolimus to Sirolimus Serum Pools on Sirolimus Concentrations Measured by Architect Immunoassay

Specimen	Sirolimus (ng/mL), Mean (SD)	Sirolimus+Everolimus (ng/mL)
Sirolimus pool 1	10.7 (0.16)	10.7
+ 3 ng/mL Everolimus	12.5 (0.21)	13.7
+ 8 ng/mL Everolimus	16.3 (0.44)	18.7
+ 16 ng/mL Everolimus	22.7 (0.58)	26.7
Sirolimus pool 2	7.2 (0.07)	7.2
+ 3 ng/mL Everolimus	9.0 (0.09)	10.2
+ 8 ng/mL Everolimus	13.4 (0.02)	15.2
+ 16 ng/mL Everolimus	19.8 (0.16)	23.2

of sirolimus and everolimus (Table 3). Therefore, for these patients, only chromatographic technique can be applied for therapeutic drug monitoring of everolimus.

The Architect sirolimus immunoassay is free from interference of both cyclosporine and tacrolimus. When a whole blood standard containing 1,200 ng/mL of cyclosporine was analyzed by the sirolimus assay, we observed an apparent sirolimus concentration of 0.18 ng/mL (mean of triplicate measurements), a value well below 1 ng/mL the sensitivity of the assay. Similarly, when we analyzed a whole blood standard containing 50 ng/mL of tacrolimus, the observed apparent sirolimus level was 0.22 ng/mL (mean of triplicate measurements). Again, the value is well below the sensitivity of the assay.

DISCUSSION

Because sirolimus and everolimus have similar mechanism of action, a combination of sirolimus and everolimus is not used as prophylactic therapy in transplant recipients. However, everolimus is often combined with cyclosporine and tacrolimus because lower concentration of cyclosporine or tacrolimus can be used. As mentioned earlier immunoassays from several diagnostic companies are available for cyclosporine, tacrolimus, and sirolimus for therapeutic drug monitoring and such tests are offered in most hospital laboratories. Because neither cyclosporine nor tacrolimus interfere with the Architect sirolimus immunoassay, it is possible to estimate everolimus concentration from apparent sirolimus concentration and either mathematical equation described in this paper. If a hospital offering transplant service or if a physician in the hospital following up a organ recipient receiving everolimus, a quick indirect determination of everolimus using sirolimus immunoassay and mathematical equation may provide useful information during a clinic visit and then the clinician can wait few days for the true everolimus concentration determined by a reference laboratory using the LC/MS for proper documentation

in the patient's chart. Because Architect sirolimus assay is free from interferences of both cyclosporine and tacrolimus, for a patient receiving everolimus along with cyclosporine or tacrolimus, our approach can be used to calculate everolimus concentration. However, if a patient is switched from sirolimus to everolimus our approach is unable to determine true everolimus concentration, and only a rough estimation of combined sirolimus and everolimus concentration can be estimated using Architect sirolimus immunoassay.

Currently, for therapeutic drug monitoring of everolimus only LC/MS methods are available (10–12) and only one FDA approved immunoassay. At present only few reference laboratories are offering therapeutic drug monitoring of everolimus. We conclude that it is possible to estimate everolimus concentration by determining apparent sirolimus concentration using Architect sirolimus immunoassay and either a polynomial equation or a linear equation.

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