Mathematical Equations to Calculate True Mycophenolic Acid Concentration in Human Plasma by Using Two Immunoassays With Different Cross-Reactivities With Acyl Glucuronide Metabolite: Comparison of Calculated Values With Values Obtained by Using an HPLC-UV Method

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Background: Both immunoassays and chromatographic methods are available for therapeutic drug monitoring of mycophenolic acid (MPA). Although chromatographic methods are more precise, immunoassays are widely used in clinical laboratories due to ease of adopting such assays on automated analyzers. We studied the possibility of using mathematical equations to calculate true MPA concentration by accounting for acyl glucuronide cross-reactivities with immunoassays by using two immunoassays with widely different cross-reactivities with the metabolite. **Methods:** We determined MPA concentrations in 20 specimens obtained from transplant recipients using cloned enzyme donor immunoassay (CEDIA) assay and a new particle enhanced turbidimetric inhibition immunoassay (PETINIA) assay. Then we developed mathematical equations to calculate true MPA concentration using values obtained

by both immunoassays and reported cross-reactivity of acyl glucuronide with respective immunoassays. Calculated concentrations were compared with values obtained by using a high-performance liquid chromatography combined with ultraviolet detection (HPLC-UV) method. **Results:** We obtained good correlation between calculated MPA concentrations and corresponding MPA level obtained by using HPLC-UV method. Using x-axis as the MPA concentrations determined by the HPLC-UV method and y-axis as the calculated MPA level, we observed the following regression equation: $y = 1.083x - 0.0995$ ($r = 0.99$, $n = 20$). **Conclusions:** Mathematical equations can be used to calculate true MPA concentrations using two immunoassays with different cross-reactivities with acyl glucuronide metabolite. J. Clin. Lab. Anal. 27:290–293, 2013. © 2013 Wiley Periodicals, Inc.

Key words: mycophenolic acid; CEDIA; PETINIA; HPLC-UV; mathematical equations

INTRODUCTION

Mycophenolic acid (MPA), an immunosuppressant is administered as a prodrug mycophenolate mofetil (brand name CellCept), the 2-morpholinoethyl ester of MPA because MPA has poor bioavailability (1). After oral administration, mycophenolate mofetil is rapidly and completely absorbed, and is quickly de-esterified in the blood and tissues into the pharmacologically active MPA (2). Majority of the drug (>99%) can be found in the plasma compartment and therapeutic drug monitoring can be conducted using plasma or serum specimen. MPA has an elimination half-life of 8–18 hours and is conjugated

with glucuronic acid in the liver to form the primary inactive metabolite, 7-O-glucuronide MPA (3). Another metabolite is acyl glucuronide but this metabolite is pharmacologically active (4). Suggested therapeutic range of

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Fig. 1. Chemical structure of mycophenolic acid.

MPA (trough serum or plasma level) is 1.0–3.5 $\mu\text{g/ml}$ (5). Chemical structure of MPA is given in Figure 1.

MPA in serum or plasma can be determined by using high-performance liquid chromatography combined with ultraviolet detection (HPLC-UV) or liquid chromatography combined with mass spectrometry. In 2010, FDA approved the CEDIA (cloned enzyme donor immunoassay) assay for MPA marketed by Thermo Scientific (Fremont, CA) and recently Siemens Diagnostics (Newark, DE) marketed a new PETINIA (particle enhanced turbidimetric inhibition immunoassay) assay for MPA (Flex reagent cartridge) for application on the Dimension analyzers. However, there are other commercially available immunoassays for MPA, for example, the EMIT (enzyme multiplied immunoassay) assay. Although immunoassays are widely used in clinical laboratories for ease of automation and speed, chromatographic methods are considered as the gold standard because MPA acyl glucuronide metabolite may cross-react with immunoassays. However, chromatographic methods are usually available only in relatively small number of hospital laboratories (academic medical centers, reference laboratories, and hospital laboratories of major hospitals) because chromatographic methods are complex requiring specially trained medical technologists. Therefore, we explored the possibility of using two immunoassays for MPA with different cross-reactivities toward acyl glucuronide metabolite to obtain two different values of MPA and then using mathematical equations to correct contribution of acyl glucuronide in total MPA measurement in order to calculate true MPA concentration. Here we report our findings.

MATERIALS AND METHODS

The CEDIA MPA assay kits, calibrators, and controls were obtained from Thermo Scientific and assays were run using a Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, IN). The PETINIA MPA assay kits and calibrators were obtained from Siemens Diagnostics and assays were run using a Dimension EXL analyzer also obtained from Siemens Diagnostics. High-performance liquid chromatography system (Alliance 2695 HPLC sys-

tem) was purchased from Waters Corporation (Milford, MA). This HPLC system is coupled with an ultraviolet detector (UV detector Model 486), which was also purchased from Waters Corporation. The HPLC column was a C-18 reverse phase column (30 $\times$ 100 mm, particle size 5 μm). All solvents used were HPLC grade. For this study, sera from 20 transplant recipients were used. These serum specimens are routinely submitted to our laboratory for therapeutic drug monitoring of MPA. After running the tests and reporting the results, these specimens are stored for a week and then discarded. This study was performed using left-over discarded specimens after deidentification according to our Institutional Review Board's approved protocol for research using left-over deidentified specimens.

The HPLC-UV analysis of MPA was accomplished by using a linear gradient of mobile phase A (methanol) and mobile phase B (0.01 M phosphate buffer at pH 3) with initial mobile phase A to B ration of 35% A and 65% B and the run time of 21 min. The HPLC column was heated at 40°C and UV detector was set at 254 nm. MPA carboxy butoxy ether was used as the internal standard. MPA controls and calibrators for HPLC-UV method were prepared in house by dissolving specific amounts of MPA in aliquots of drug-free plasma. Stock solution of MPA was prepared by dissolving 20 mg of MPA in 5 ml of methanol and then working solutions were prepared by proper dilution of the stock solution. However, working solution of the internal standard was prepared in acetonitrile, the extraction solvent. We used nine calibrators with concentrations of 0.0 $\mu\text{g/ml}$ (zero calibrator), 0.2 $\mu\text{g/ml}$, 0.4 $\mu\text{g/ml}$, 1.0 $\mu\text{g/ml}$, 2.0 $\mu\text{g/ml}$, 5.0 $\mu\text{g/ml}$, 10.0 $\mu\text{g/ml}$, 20.0 $\mu\text{g/ml}$, and 40.0 $\mu\text{g/ml}$ for developing calibration curve. Three controls (0.5 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, and 12.5 $\mu\text{g/ml}$) were used for quality control. The assay was linear for MPA concentration between 0.2 and 40 $\mu\text{g/ml}$. This method was adopted from the report of Patel et al. with modification (6). There are also other published reports in the literature for analysis of MPA using HPLC-UV and any such method is suitable for routine therapeutic drug monitoring of MPA in a toxicology laboratory (7–10).

The CEDIA MPA assay was run using a Hitachi 917 analyzer and parameters provided by the manufacturer (Thermo Scientific). This assay uses recombinant DNA technology. The assay is based on the enzyme β -galactosidase, which has been genetically engineered into two inactive fragments termed as enzyme donor and enzyme acceptor. These fragments spontaneously combine together to form active enzyme that cleaves a substrate generating color. The assay analyte (MPA) competes with analyte conjugated to enzyme donor part of β -galactosidase for limited number of antibody binding sites. The amount of active enzyme formed (if analyte is present in the sample, then it binds with the antibody

leaving the conjugated part of enzyme donor molecule to combine with enzyme acceptor part of the molecule) and the resultant color change is directly proportional to the concentration of MPA in the serum specimen. The assay has an analytical measurement range of 0.3–10 µg/ml.

The PETINIA assay uses a monoclonal antibody against MPA. This assay was run on the Dimension EXL analyzer using parameters supplied by the manufacturer. The calibrators for PETINIA assay was provided by the manufacturer but controls were not available. Therefore, we used control available from Thermo Scientific for quality control of PETINIA assay. This assay uses five calibrators with an analytical measurement range of 0.2–30 µg/ml.

RESULTS

The between run precisions of low and high control of HPLC-UV reference method were <7% while the between run precisions of low and high control for the PETINIA assay were <5%. However, between run precisions low and high controls for the CEDIA assay was <10% in our laboratory.

The average observed bias between the CEDIA MPA assay and the HPLC-UV method was 15.6% in our laboratory but occasionally more than 20% bias may be observed in MPA level determined by the CEDIA assay compared to the HPLC-UV method in some transplant recipients, especially liver transplant recipients. In this study we selected sera from 20 transplant patients where the MPA concentrations determined by the CEDIA assay were more than 20% than the corresponding values obtained by the HPLC-UV method. For these 20 specimens MPA concentrations were also determined by the PETINIA assay.

The reported cross-reactivity of acyl glucuronide with the CEDIA assay is 158% while the reported cross-reactivity of acyl glucuronide with the PETINIA assay is 52% (Package inserts). Therefore assuming the true MPA concentration as “x” and acyl glucuronide concentration as “y,” we can construct two simultaneous equations:

$$x + 1.58 y = \text{CEDIA mycophenolic acid concentration} \quad (1)$$

$$x + 0.52 y = \text{PETINIA mycophenolic acid concentration} \quad (2)$$

Solving these two equations:

$$1.58 y - 0.52 y = \text{CEDIA value} - \text{PETINIA value}$$

$$\text{Therefore: } 1.06 y = \text{CEDIA value} - \text{PETINIA value}$$

TABLE 1. Calculated and Observed Mycophenolic Acid (MPA) Concentrations in 20 Transplant Recipients

Patient ID	Observed MPA levels			Calculated levels	Bias ^a
	PETINIA	CEDIA	HPLC-UV		
1	1.1	2.0	0.8	0.7	− 12.5%
2	6.9	7.8	6.0	6.5	+8.3%
3	7.5	7.9	6.6	7.3	+11.6%
4	1.9	2.5	1.7	1.6	− 5.9%
5	2.9	3.8	2.6	2.5	− 3.8%
6	4.7	5.2	4.2	4.5	+7.1%
7	6.5	7.8	5.3	5.8	+9.4%
8	1.1	1.7	0.8	0.8	None
9	2.2	2.9	1.8	1.8	None
10	5.2	7.7	3.8	4.0	+5.2%
11	1.7	2.0	1.5	1.6	+6.7%
12	5.0	5.6	4.5	4.7	+4.4%
13	2.2	2.7	1.9	2.0	+5.2%
14	1.3	2.3	0.8	0.8	None
15	1.5	2.4	1.2	1.1	− 8.3%
16	1.0	2.0	0.5	0.5	None
17	1.1	2.1	0.6	0.6	None
18	4.2	4.9	3.8	3.9	+2.6%
19	1.9	2.1	1.7	1.8	+5.9%
20	1.5	1.7	1.3	1.4	+7.7%

^aBias was calculated by formula: (HPLC value − Calculated value/HPLC value) × 100.

After obtaining the value of “y,” value of “x” can be easily calculated from Equation (1)

For example, in patient 1, MPA concentration determined by the CEDIA assay was 2.0 µg/ml while the corresponding value obtained by the PETINIA assay was 1.1 µg/ml.

Therefore, Equation (1): $x + 1.58 y = 2.0$

Equation (2): $x + 0.52 y = 1.1$

Therefore: $1.06 y = 0.9$, or $y = 0.9/1.06 = 0.85$

Putting value of y in Equation (1), we get : $x + 1.58 \times 0.85 = 2.0$

Therefore, $x + 1.34 = 2.0$

Or calculated value of MPA “x” is $2.0 - 1.34 = 0.66$, which can be expressed as 0.7 µg/ml, using one decimal place. The MPA concentration obtained by using HPLC-UV method in this patient was 0.8 µg/ml, which was in good agreement with the calculated MPA concentration of 0.7 µg/ml. We also observed good agreement between calculated MPA concentration and observed MPA concentration in other 19 patients we studied (Table 1). Using x-axis as the MPA concentration determined by using the HPLC-UV method and y-axis as calculated MPA concentrations, we observed the following regression equation (Fig. 2):

$$y = 1.083x - 0.0995 (r = 0.99, n = 20)$$

Fig. 2. Regression analysis showing correlation between mycophenolic acid (MPA) concentrations obtained by using HPLC-UV method and calculated MPA concentration.

DISCUSSION

The regression analysis indicated that there was a good correlation between calculated MPA concentrations and MPA concentrations determined by HPLC-UV method. Initially all 20 specimens showed more than 20% positive bias in MPA concentrations determined by the CEDIA assay compared to the HPLC-UV method. The highest positive bias between calculated MPA concentrations and measured by HPLC-UV method was 12.5%. No bias was observed between calculated MPA level and MPA concentration determined by HPLC-UV in 5 out of 20 specimens we analyzed.

Significant positive bias in the CEDIA MPA assay has been reported before. Westley et al. also however observed an overall 18% in the CEDIA MPA assay compared to a reference HPLC-UV method (11). Positive bias has also been reported in the EMIT assay for MPA (12). Therefore, major limitation of using immunoassays for therapeutic drug monitoring of MPA is significant metabolite cross-reactivity.

Although chromatographic methods are superior to immunoassays for therapeutic drug monitoring of MPA, majority of small- and medium-size hospitals do not have chromatographic methods available in their hospital laboratories. However, adopting another immunoassay for measuring MPA is a valid alternative to acquiring chromatographic analyzers. Our mathematical approach to calculate MPA level based on MPA concentration determined by two different immunoassays may be an alternative for a hospital laboratory where chromatographic method is not available. If a clinician questions validity of an MPA level as determined by an immunoassay and if all quality control values are within limit, then true MPA concentration may be calculated by using our approach.

CONCLUSION

The mathematical approach presented in this paper to calculate MPA level may be useful for clinical laboratories that do not have chromatographic method available. However, after calculating true MPA concentration using these equations, it is important to send a specimen to a reference laboratory for measuring MPA level using a specific chromatographic method in order to document the true MPA level in the patient's chart. This will be very useful in a medical-legal scenario if the validity of calculated MPA level faces any legal challenge in a court of law in future.

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