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Original Article

Therapeutic Monitoring of Mycophenolic Acid in Renal Transplanted Patients by a Validated HPLC Method

Mikaela Kolaci[1], Leonard Deda[2], Alma Idrizi[3], Myftar Barbullushi[3], Dariel Thereska[4]

[1] *National Agency of Medicines and Medical Devices, Tirana, Albania*

[2] *Department of Pharmacology, Faculty of Medicine, University of Medicine, Tirana, Albania*

[3] *Nephrology-Dialysis-Transplantation Department, Mother Theresa University Hospital Center, Tirana, Albania*

[4] *Department of General Surgery, Faculty of Medicine, University of Medicine, Tirana, Albania*

Corresponding author: Mikaela Kolaci, National Agency of Medicines and Medical Devices, Rr. "Reshit Collaku" 23/1, Tirana, Albania; E-mail: mikaela_kolaci@yahoo.com; Tel.: 00355695303079

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Abstract

Introduction: Mycophenolate mofetil and its active metabolite mycophenolic acid are routinely used as immunosuppressant drugs in solid organ transplantation in a fixed daily dose regimen in association with cyclosporine, tacrolimus and steroids. Therapeutic drug monitoring for mycophenolic acid concentration has been suggested to optimize outcomes by reducing rejection and drug related toxicities in clinical renal transplantation.

Aim: To determine the predose concentration of mycophenolic acid in renal transplanted patients by a validated proposed high-performance liquid chromatography (HPLC) method and to estimate the interindividual variability based on the therapeutic target.

Materials and methods: An HPLC method combined with protein precipitation has been validated for mycophenolic acid determination in the human plasma obtained from 21 renal transplant recipients. HPLC analysis was carried out using the chromatographic system Agilent Technologies 1200 DAD. Samples were injected manually, and the compounds were separated on a LiChrosphere® select B C18 analytical column. The mobile phase was 45:55 (v/v) acetonitrile-buffer phosphate, pH 2.5, flow rate of 1.0 mL/min and column temperature of 30°C. Detection was performed at 215 nm. Whole blood samples were collected into vacutainers containing EDTA and separated at 6000 g for 10 minutes. A 200-µL aliquot of patient plasma was transferred to a tube, followed by addition of 10 µL of naproxen as internal standard and 400 µL of acetonitrile (v/v) as a protein precipitating agent. Each tube was vortex-mixed for 30 sec and then centrifuged for 10 min at 10000 rpm. 20 µL of the supernatant was injected into the HPLC system for analysis.

Results: The method showed appropriate linearity for MPA with correlation coefficient greater than 0.999. High inter-patient variability is observed with 18% of patients within the target trough concentration range, 27% of patients below the target trough concentration range and 54% over the range with risk of toxicity.

Conclusions: Therapeutic monitoring of MPA might contribute to a better management of renal transplant recipient with the goal of optimizing therapeutic regimens in order to reduce the risk of rejection and MPA-related toxicity.

Keywords

immunosuppressive drugs, interindividual variability, predose concentration, protein precipitation

INTRODUCTION

Mycophenolate mofetil (MMF) is an immunosuppressive agent used in renal, heart, and liver transplantation for the prevention of acute rejection. MMF is used at a fixed-dose regimen of 1 g p.o. BID in renal allograft recipients in association with cyclosporine, tacrolimus, and steroids.[1] Mycophenolate mofetil (MMF) is a weak base, exhibiting a strong pH-dependent solubility profile (solubility decreases when pH increases). It is absorbed rapidly and is hydrolyzed by esterases to the active metabolite mycophenolic acid (MPA).[2]

To prevent gastrointestinal adverse events, which are frequently seen during MMF treatment, enteric-coated mycophenolate sodium (EC-MPS) was developed. EC-MPS and MMF are both prodrugs of mycophenolic acid (MPA) that showed similar efficacy and safety profiles and are alternatively used as immunosuppressive agents in de novo and stable kidney transplantation recipients.[3] The free fraction of the highly protein bound MPA (97%) is thought to be responsible for the immunosuppressive effect.[4] MPA is primarily metabolized by glucuronidation of the phenolic hydroxy group by uridine diphosphate-glucuronosyltransferases (UGTs) to an inactive mycophenolic acid glucuronide (MPAG), which is the major urinary excretion product of MPA.[5]

Shaw et al.[6] reported that the area under the curve time-concentration (AUC) for MPA varies as much as tenfold between patients receiving the same dose of MMF. In addition, within subject changes in the AUC for MPA have been noted over time following transplantation. The inter-patient variability in MPA exposure is wide compared with the therapeutic window[7] and is influenced by many factors like coadministration of cyclosporine, low plasma albumin levels, and impaired renal function.[8] The incidence of biopsy-proven acute rejection is correlated with the MPA AUC and the MPA predose level, of which AUC showed the best correlation.[9] However, AUC measurements are more complicated to perform for practical reasons.[10] Furthermore, MMF can produce hematologic and/or gastro-intestinal toxicity. Mourad et al.[11] reported pharmacokinetic/pharmacodynamic relationship between MPA and clinical events.

Therapeutic drug monitoring (TDM) for mycophenolic acid (MPA) levels in renal transplantation by using different analytical methods has been suggested to optimize outcomes by reducing rejection or drug related toxicities.[12] Targeting mycophenolic acid trough plasma concentration in the range 1.6 mg/L to 3 mg/L have been suggested to limit toxicity.[13]

Several HPLC-UV[14-21] methods in combination with protein precipitation have been described in the current literature for the determination of MPA and its metabolites in human plasma.

AIM

The aim of the present study was to validate the proposed HPLC method and to apply it for the determination of mycophenolic acid predose concentration obtained from renal transplanted recipients. The described HPLC method combined with protein precipitation for the determination of MPA in human plasma is rapid and reliable. The sample preparation is simple and can be performed in every laboratory. Determination of predose concentration of mycophenolic acid could help the physician to estimate if the concentration is within the target range and consequently adapt the dose when necessary.

MATERIALS AND METHODS

MPA (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phtalanyl) -4-methyl-4-hexenoic acid) was kindly provided by the representative office of the pharmaceutical company Novartis (Tirana, Albania). Naproxen as solid standard compound was used as internal standard.

Acetonitrile HPLC was purchased from Sigma-Aldrich. Ortho-phosphoric acid analytical reagent of pH=2.5 was of the grade suitable for HPLC. HPLC analysis was carried out using the chromatographic system Agilent Technologies 1200 DAD equipped with on-line degasser, binary pump, column oven, and photo diode array detector. The HPLC column used was LiChrospher® select B C18 analytical column (particle size 5 μm) with precolumn guard. The water for chromatography was purified. Before use, the mobile phase was degassed and purified by vacuum filtration through 0.45 μm Millipore filters.

20 μL of the sample was injected manually into the chromatographic system Agilent Technologies 1200 DAD. Data were collected from the Agilent's ChemStation software. Statistical analysis was carried out by using the Microsoft Excel software.

The mobile phase was 45:55 (v/v) acetonitrile-buffer phosphate (pH equal to 2.5 adjusted with 1 M orthophosphoric acid). The flow rate was 1.0 mL/min and column temperature was set at 30°C. Detection was performed at 215 nm.

Whole blood samples were collected from twenty-one renal transplant recipients during their monthly routine visit at the Transplantation Unit of University Hospital Mother Theresa in Tirana. The whole blood sample to be analyzed for MPA predose level was collected into vacutainers containing EDTA and separated at 6000 g for 10 minutes. All plasma samples were stored at −20°C until analysis.

A 200-μL aliquot of patient plasma was transferred to a tube, followed by addition of 10 μL of naproxen (from a solution of 0.5 mg/ml in acetonitrile) as internal standard and 400 μL of acetonitrile (v/v) as a protein precipitating agent. Each tube was vortex-mixed for 30 sec and then centrifuged for 10 min at 10000 rpm. 20 μL of the supernatant was injected into the HPLC system for analysis.

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Stock solution for the construction of the standard curve of MPA was prepared by dissolving the MPA in methanol to yield concentration of 1.0 mg/ml. Working standard solutions of MPA (0.5, 1.0, 2.0, 4.0 and 8.0 μg/mL) were prepared by serial solution with water. Stock solutions were stored at 2–8°C. Blank plasma samples were treated as described above, spiked with the working standard solutions and 10 μL of naproxen (from 0.5 mg/ml solution) as internal standard. The calibration curve was constructed from the peak-height ratio of the MPA to the naproxen internal standard from the HPLC chromatograms and then plotted against the nominal MPA concentration.

Inter-day and intra-day precision and accuracy were evaluated from the analysis of control samples (low QC of 1 μg/mL, medium QC of 5 μg/mL and high QC of 10 μg/mL) measured on five different days. Inter-day precision and accuracy were evaluated by analyzing spiked plasma samples five times over the course of the one day in random order. Precision of the HPLC method at each concentration was determined by comparing the coefficient of variation (CV) with the accuracy estimated for each spiked control.

RESULTS

This HPLC-UV method combined with protein precipitation for the determination of MPA concentration in human plasma is simple and suitable to be used in any laboratory for therapeutic drug monitoring. Typical chromatograms obtained from blank plasma and plasma spiked with MPA concentration of 1 μg/ml and 10 μL of naproxen as internal standard are shown in **Figs 1A** and **1B**, respectively. Reten-

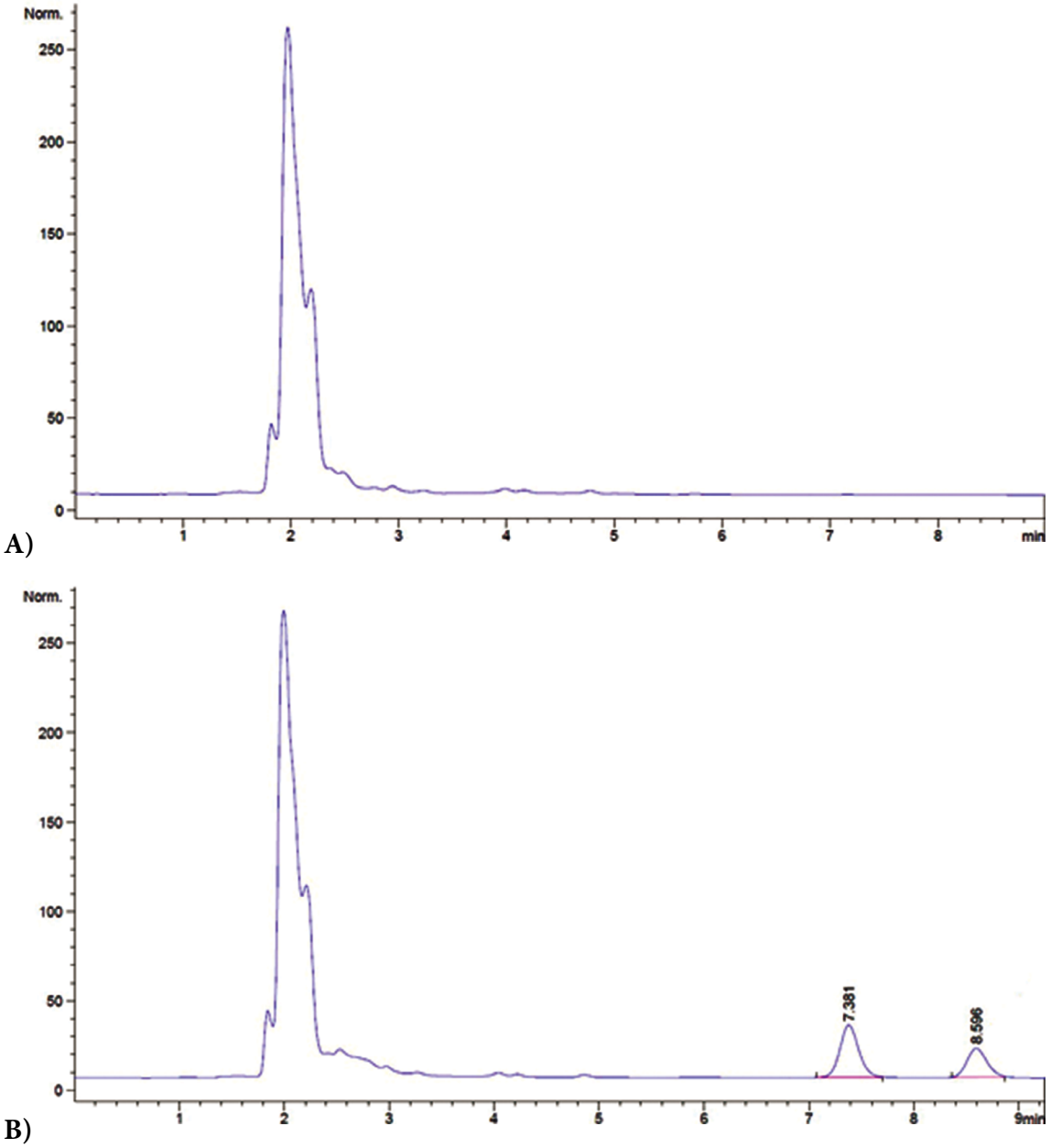

Figure 1. A) Blank plasma; **B**) Plasma spiked with MPA and naproxen as internal standard.

tion times for MPA was about 7 min and for the internal standard about 8 min. This method requires 200 µl of plasma and takes 10 min for a single chromatographic run.

The calibration curve for MPA plasma was found to be linear over the concentration range of 0.5–8 µg/mL. The typical calibration curve was obtained:

$y=0.0806x + 0.0151$; $r^2 = 0.999$

where y is the peak area ratio, x is the concentration of the compound, and *r* is the correlation coefficient (**Fig. 2**).

The coefficients of variation (CV) and accuracy for intra- and inter-day assays were determined at Quality control concentrations of 1 – 10 µg/mL for MPA. CV values and accuracies for intra- and inter-day assays are shown in **Table 1**. The precision and accuracy of this HPLC assay is suitable for routine therapeutic drug monitoring applications.

This study enrolled twenty-one renal transplant recipients under treatment with mycophenolate mofetil (Cellcept®) and EC-MPS (Myfortic®) followed at the Transplantation Unit of the Nephrology Service at the University Hospital of Mother Theresa, Tirana, Albania. It was the first time that therapeutic monitoring of MPA was performed in this Hospital Center. Whole blood samples were obtained in EDTA tube after informed consent to the patients during their monthly check-up at the Transplantation Unit. The patients were receiving tacrolimus or cyclosporine and prednisolone for immunosuppression. In the present method, no analytical interference was detected for these compounds. The results of the assayed plasma from twenty-one renal transplant recipients under treatment with MMF and EC-MPS are displayed in **Table 2**.

The MPA concentrations in renal transplant recipients ranged from 0.46 µg/mL to 16.59 µg/mL.

High inter-patient variability is observed with 18% of patients within the target trough concentration range, 27% of patients below the target trough concentration range and 54% over the range with risk of toxicity.

DISCUSSION

The proposed HPLC-UV method combined with protein precipitation has been validated for the analysis of MPA in human plasma from renal transplanted recipients. Twenty-one renal transplant recipients were enrolled in the study. Their predose concentration was measured using the described method. The method showed appropriate linearity for MPA with correlation coefficient greater than 0.999. No interferences were observed during the analysis of patients' plasma. Therapeutic monitoring of MPA has been suggested to optimize therapy in renal transplantation. As it has been previously reported, high inter-patient varia-

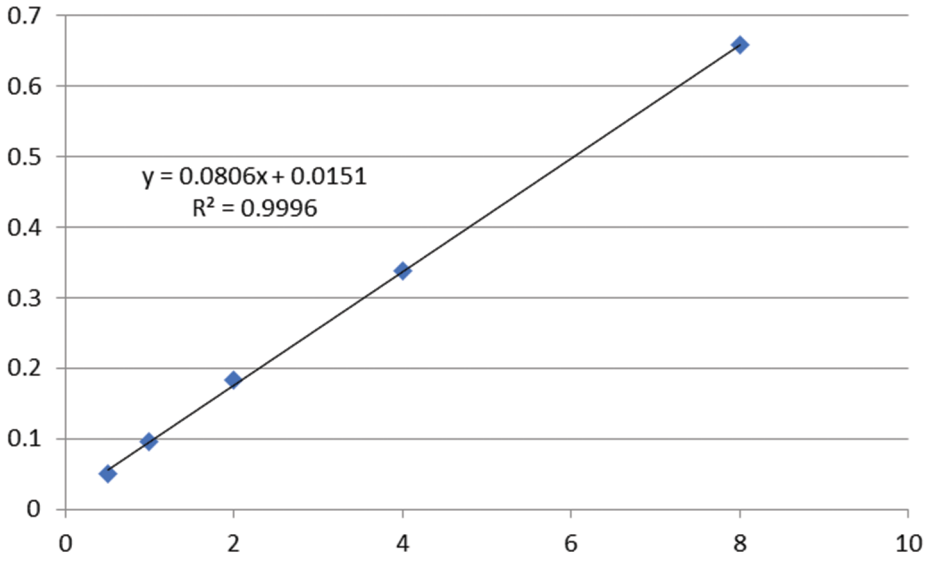

Figure 2. Calibration curve of mycophenolic acid (n=5). X axis: standard MPA concentration (0.5–8 µg/ml); Y axis: peak area ratio.

Table 1. Accuracy and precision of HPLC assay for the determination of MPA in human plasma (n=5)

Added µg/ml	Intra-day			Inter-day		
	Found mean ±SD	Precision (%)	Accuracy (%)	Found mean ± SD	Precision (%)	Accuracy (%)
1	1±0.04	4.3%	4.5%	0.9±0.1	12.1%	12.1%
5	5.1±0.1	1.5%	1.5%	5.2±0.3	5.7%	4.7%
10	10±0.4	4.2%	3.7%	9.9±4.6%	4.6%	4.1%

Table 2. Results of the assay of plasma of renal transplant recipients under treatment with MMF or EC-MPS (n=5)

Patient	Dose of MMF mg/d	Dose of EC-MPS mg/d	Mean concentration of MPA in plasma µg/mL
1		720	1.66
2		1440	1.83
3		720	2.79
4	1000		1.20
5		720	3.47
6		1440	3.18
7		1440	5.37
8		1440	3.53
9		1080	0.46
10		720	7.32
11	1500		3.43
12		1440	1.19
13		1440	3.72
14		720	13.83
15		1080	16.59
16		720	3.94
17		720	1.55
18		720	1.29
19	2000		3.35
20	4000		1.09
21	1000		4.13

bility is observed in our study. Determination of predose concentration in the randomly selected patients revealed that only 18% of the analyzed patients had concentrations within the target concentration range, 27% of patients below the target concentration range and 54% over the range. Dose is a poor predictor for MPA exposure due to high within-patient variability. For this reason, it is important to monitor predose concentrations especially those below the target range as this can lead to rejection episodes. On the other hand, more than half of the patients present high concentrations of MPA with the risk of hematological and gastrointestinal side effects. In this context, we recommend therapeutic monitoring of mycophenolic acid to optimize the dosage regimen. As samples were taken randomly, we suggest considering different factors while evaluating the data, like time of transplantation, co-medication, albumin levels and renal function. In addition, our results are limited to one single measurement. Monitoring of predose levels in different days for the same patient could be helpful in order to estimate the need for dose adaptation.

CONCLUSIONS

The proposed HPLC method combined with protein precipitation is suitable for MPA analysis in the plasma obtained from renal transplant recipients on polytherapy due to no analytical interferences. Therapeutic monitoring of MPA might contribute to a better management of renal transplant recipient with the goal of optimizing therapeutic regimens to reduce the risk of rejection and. MPA related toxicity.

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Терапевтический мониторинг микофеноловой кислоты у пациентов с пересаженной почкой с помощью проверенного метода ВЭЖХ

Микаела Колаци[1], Леонард Деда[2], Алма Идризи[3], Мифтар Барбулуши[3], Дариел Тереска[4]

[1] *Национальное агенство лекарств и медицинской техники, Тирана, Албания*

[2] *Кафедра фармакологии, Медицинский факультет, Медицинский университет, Тирана, Албания*

[3] *Отделение нефрологии-диализа-трансплантации, Университетская больница „Мать Тереза", Тирана, Албания*

[4] *Кафедра общей хирургии, Медицинский факультет, Медицинский университет, Тирана, Албания*

Адрес для корреспонденции: Микаела Колаци, Национальное агенство лекарств и медицинской техники, ул. „Решит Цолаку" 23/1, Тирана, Албания; E-mail: mikaela_kolaci@yahoo.com; Тел.: 00355695303079

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Резюме

Введение: Микофенолятмофетил и его активный метаболит микофеноловая кислота обычно используются в качестве иммунодепрессантов при трансплантации твёрдых органов в режиме фиксированной суточной дозы вместе с циклоспорином, такролимусом и стероидами. Было высказано предположение, что мониторинг терапевтического препарата на предмет концентраций микофеноловой кислоты оптимизирует результат за счёт уменьшения отторжения и интоксикации, связанной с лекарством, при клинической трансплантации почек.

Цель: Определить концентрацию микофеноловой кислоты перед дозированием у пациентов с трансплантатом почки с помощью проверенного метода высокоэффективной жидкостной хроматографии (ВЭЖХ) и оценить межличностную изменчивость на основе терапевтической цели.

Материалы и методы: ВЭЖХ была подтверждена в сочетании с осадком белка для определения микофеноловой кислоты в плазме человека, взятой у 21 реципиента трансплантата почки. Анализ ВЭЖХ проводили с использованием хроматографической системы Agilent Technologies 1200 DAD. Образцы вводили вручную, и соединения разделяли с помощью аналитической колонки LiChrosphere® select B C18. Подвижная фаза представляла собой фосфат ацетонитрильного буфера 45:55 (v/v), PH 2.5, скорость инфузии 1.0 mL/min и температура колонки 30° C. Обнаружение производилось при 215 nm. Образцы цельной крови отбирали в вакуутайнеры с раствором EDTA и разделяли на 6000 g в течение 10 минут. Аликвоту плазмы пациента объемом 200-μL переносили в пробирку, затем добавляли 10 μL напроксена в качестве внутреннего стандарта и 400 μL ацетонитрила (v/v) в качестве агента, осаждающего белок. Каждую пробирку встряхивали в течение 30 секунд. и затем центрифугировали 10 минут при 10000 rpm. 20 μL супернатанта вводили в систему анализа ВЭЖХ.

Результаты: Метод показал подходящую линейность IFC с коэффициентом корреляции выше 0.999. Более высокая вариабельность между пациентами наблюдалась у 18% пациентов в целевом диапазоне минимальных концентраций, у 27% у пациентов ниже целевого диапазона минимальных концентраций и у 54% пациентов выше диапазона риска интоксикации.

Заключение: Терапевтический мониторинг MPA может способствовать лучшему ведению реципиента почечного трансплантата с целью оптимизации терапевтических режимов для снижения риска отторжения и интоксикации, связанной с MPA.

Ключевые слова

иммунодепрессанты, индивидуальная вариабельность, концентрация перед дозированием, преципитация белков