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High-Performance Liquid Chromatography Method Appropriate for the Determination of Mycophenolic Acid in Renal Transplantation

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Abstract

Background: Mycophenolate mofetil and mycophenolate sodium, both produgs of the active metabolite mycophenolic acid, are immunosuppressive agents used in transplantation for the prevention of acute rejection. The inter-patient variability in mycophenolic acid exposure is wide compared with the therapeutic window. Therapeutic drug monitoring for mycophenolic acid levels in renal transplantation has been suggested to optimize outcomes by reducing rejection or drug related toxicities.

Aim: The aim of this study is to validate a simple, rapid and sensitive high-performance liquid chromatography method combined with protein precipitation for the determination of the concentration of mycophenolic acid in human plasma.

Method: HPLC analysis was carried out using the chromatographic system Agilent Technologies 1200 DAD. Precipitation of plasma proteins was performed by the addition of acetonitrile. Samples were injected manually and the compounds were separated on a Lichrosphere select B C18 analytical column (particle size 5μm). The mobile phase consisted of 5:55 (v/v) acetonitrile-buffer phosphate adjusted at pH 2.5, flow rate was 1.0mL/min and column temperature was kept at 30°C. Detection was performed at 215nm. Naproxen was used as

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internal standard. 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. The precision and accuracy of this HPLC assay were estimated.

Results: The proposed method showed appropriate linearity for mycophenolic acid (MPA) with correlation coefficient greater than ($r2 > 0.999$). The precision and accuracy of intra-day and inter-day of this HPLC assay is suitable for routine therapeutic drug monitoring applications. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be respectively 0.1 μg/ml and 0.4 μg/ml.

Conclusion: This HPLC-UV method for the determination of MPA concentration in human plasma is simple and suitable to be used for therapeutic monitoring. The method was intended to be applied in the analysis of human plasma from renal transplanted patients followed at the University Hospital Center "Mother Theresa" in Tirana, Albania.

Keywords: mycophenolic acid, HPLC method, renal transplantation

INTRODUCTION

Mycophenolate mofetil (MMF) is an immunosuppressive drug used to prevent rejection following solid organ transplantation. MMF is used at fixed-dose regimen of 1 g per os twice daily in renal allograft recipients in association with cyclosporine, tacrolimus and steroids (1). The prodrug mycophenolate mofetil (Figure 1) is rapidly and completely absorbed and hydrolysed to the active compound mycophenolic acid (MPA). The free fraction of the highly protein bound MPA (97%) is thought to be responsible for the immunosuppresive effect (2). To prevent gastrointestinal adverse events, which are frequently seen during MMF treatment, enteric-coated mycophenolate sodium (EC-MPS, Myfortic ®) was developed. EC-MPS and MMF are both prodrugs of MPA that showed similar efficacy and safety profiles and are alternatively used as immunosuppressive agents in de novo and stable kidney transplantation recipients (3). MPA potently, selectively, and reversibly inhibits inosine monophosphate dehydrogenase (IMPDH) and therefore inhibits the de novo pathway of purine synthesis in T and B cells (T and B lymphocytes) (4,5). 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 (6).

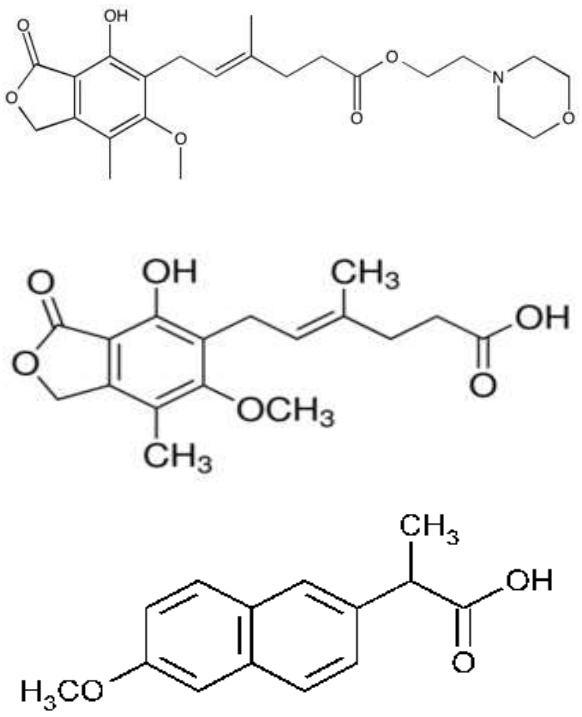

Figure 1. Structures of mycophenolate mofetil, MMF (a), mycophenolic acid, MPA (b), and naproxen internal standard, IS (c)

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). Furthermore, MMF can produce hematologic and/or gastro-intestinal toxicity. 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 (9).

Several gas chromatographic and HPLC methods have been developed for the determination of MPA. According to the current literature, HPLC-

UV (10-17) and HPLC-MC (18-23) methods in combination with protein precipitation were described for the determination of MPA and its metabolites in human plasma, serum, urine, saliva and microsomal incubations. HPLC-MS methods offer better sensitivity in comparison with HPLC-UV methods. However, HPLC-MS equipment is costly and HPLC-UV methods are thus more commonly used in clinical practice.

The proposed HPLC-UV method combined with protein precipitation is rapid and suitable to be used for the determination of MPA in human plasma. The developed method is based on simple sample preparation that can be performed in every laboratory.

The aim of the present study was to validate the proposed HPLC method in order to be applicable for therapeutic monitoring of MPA in human plasma.

MATERIALS AND METHODS

Reagents and Chemicals 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). Solid Naproxen was used as an internal standard.

Acetonitrile HPLC grade was purchased from Sigma-Aldrich.

Ortho-phosphoric acid analytical reagent to reach a certain pH suitable for HPLC grade.

HPLC system 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 Lichrosphere select B C18 analytical column (particle size 5μm) eqquiped with a precolumn guard. The water used for chromatography was previously purified. Before use, the mobile phase was degassed and purified by vacuum filtration through 0.45μm 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. Statystical analysis was carried out by using the Microsoft Excel software.

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

Sample preparation A 200 μL aliquot of blank plasma was transferred in a tube and was spiked with working standard solutions of MPA, followed by addition of 10 μL of naproxen (from a solution of 0.5mg/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.

Calibration curve 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 μg/ml) were prepared by serial dilutions with water. Stock solutions were stored at +4°C. Blank plasma samples were treated as described above, spiked with the working standard solutions and 10 μL of naproxen 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.

Assay validation 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 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. Relative recovery was estimated by the measured ratio of control samples at low QC of 1 μg/ml, medium QC of 5 μg/ml and high QC of 10 μg/ml to the aqueous solutions at the same concentrations.

RESULTS

Chromatograms 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 1μg/ml and 10μL of naproxen as internal standard are shown in Figure 2 (a) and (b), respectively. Retention times for MPA was about 7 min and for the internal standard about 8 min. This method requires 200μl of plasma. Each chromatographic run lasts 10 min.

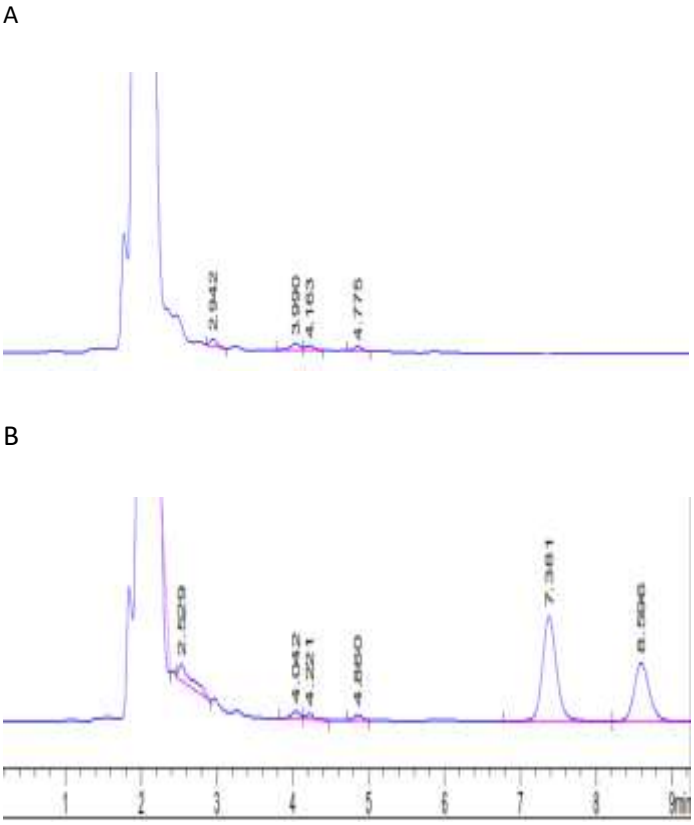

Figure 2. (a) Blank plasma (b) Plasma spiked with MPA and Naproxen as internal standard

Calibration Curve 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.

The Limit of Detection (LoD) and Limit of Quantification (LoQ) were found to be respectively 0.1 μg/ml and 0.4 μg/ml.

Precision and Accuracy 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. Precision and accuracies values 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.

Relative recovey was estimated by the measured ratio of control samples at low QC of 1 μg/ml, medium QC of 5 μg/ml and high QC of 10 μg/ml three times to the aqueous solutions at the same concentrations shown in Table 2. Recovery ranged from 90% to 93%.

Table 2. Relative recovery of MPA by the proposed HPLC method (n=3)

Added μg/ml	Recovery %	Mean (%)
1	86 92 87	91
5	110 87 76	90
10	88 96 96	93

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%

DISCUSSION

This HPLC-UV method combined with protein precipitation has been validated for the analysis of MPA in human plasma from renal transplanted recipients. The method showed appropriate linearity for MPA with correlation coefficient greater than 0.999. The proposed method is suitable for routine MPA analysis as well as pharmacokinetic studies. The determination of mycophenolic acid could help the physician assess if concentrations are within the therapeutic range, limiting toxicity and preventing rejection due to low drug concentration.

CONCLUSION

The proposed HPLC method combined with protein precipitation is suitable for MPA analysis in plasma obtained from renal transplant recipients. 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 MPA-related toxicity and prevent rejection.

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Conflict of Interest Disclosure: The authors declare that they have no conflict of interest.

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