Original article

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VALIDATION OF HPLC METHOD FOR THE DETERMINATION OF MYCOPHENOLIC ACID IN HUMAN PLASMA OBTAINED FROM RENAL TRANSPLANT RECIPIENTS

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A simple, fast and sensitive HPLC method combined with protein precipitation has been developed and validated for the determination of mycophenolic acid in human plasma, obtained from renal transplant recipients. For the analysis to be performed, the C18 Bakerbond-BDC analytical column (250 mm x 4.6 mm i.d., particle size 5 μm) was used. For chromatographic separation the optimal conditions were established with the mobile phase acetonitrile - 10 mM phosphate buffer, pH 2.5 (50:50, v/v) at the flow rate of 1.0 mL/min, temperature 30°C, and detection at 215 nm. Chromatographic run time was about 6 minutes. Precipitation of plasma proteins was performed by the addition of 0.3% trifluoroacetic acid in acetonitrile (v/v). The HPLC method combined with protein precipitation was subjected to validation. Linearity was observed over the concentration range of 0.2-100 μg/mL for mycophenolic acid with correlation coefficient 0.9995. Moreover, a good intra-day and inter-day precision was confirmed, with relative standard deviation below 8.64%, while accuracy ranged from 89.31% to 107.67% for mycophenolic acid. Finally, the method was successfully applied in the analysis of plasma samples obtained from renal transplant recipients in polytherapy. *Acta Medica Medianae 2016;55(4):28-36.*

Key words: *mycophenolic acid, human plasma, protein precipitation, high-performance liquid chromatography*

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Introduction

Mycophenolate mofetil (MMF, Figure 1a) has been used to prevent acute graft rejection after solid organ transplantation. Following oral administration, prodrug MMF is rapidly absorbed and hydrolyzed to its active metabolite mycophenolic acid (MPA, Figure 1b). In proliferating lymphocytes MPA decreases guanine nucleotide levels by selective, noncompetitive and reversible inhibition of inosine monophosphate dehydrogenase. As a result, MPA exhibits immunosuppressive activity (1).

MPA is mostly metabolized by glucuronidation to 7-O-glucuronide conjugate (MPAG), which is excreted in urine. MPAG does not exert pharmacological activity and can be hydrolyzed to MPA during enterohepatic recirculation. In addition, MPA is further metabolized to acyl glucuronide (AcMPAG), which may contribute to side effects after MMF administration (2).

In renal transplant recipients, MPA pharmacokinetics has the characteristic of large inter-individual and intra-individual variability, especially in early post-transplantation period. The pharmacokinetics of MPA is influenced by a number of factors: concomitant administration of tacrolimus or cyclosporine A, food intake, analytical technique for MPA determination, time after transplantation and pharmacogenetic factors (3). In order to perform a population pharmacokinetic study of MPA (3), a HPLC method combined with protein precipitation has been developed and validated for MPA determination in the human plasma obtained

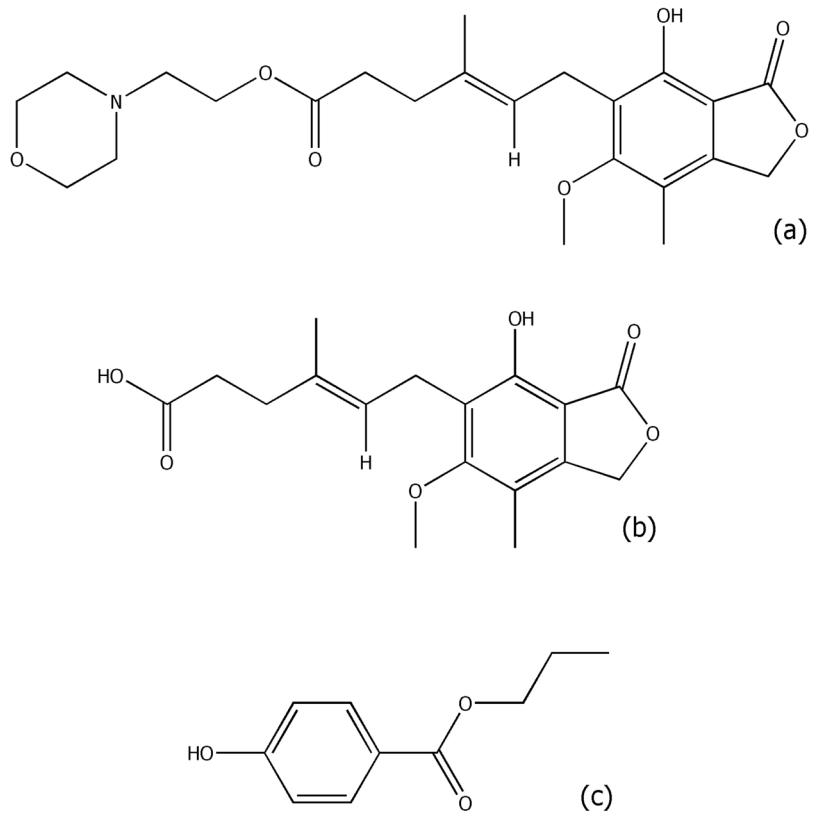

Figure 1. Structures of mycophenolate mofetil, MMF (a), mycophenolic acid, MPA (b), and propylparaben internal standard, IS (c)

from renal transplant recipents. Moreover, the proposed method could be utilized for therapeutic drug monitoring of MPA.

According to the current literature, HPLC-UV (1, 2, 4-10) and HPLC-MS (11-16) methods in combination with protein precipitation were described for the determination of MPA, its metabolites and other drugs in human plasma (1, 2, 4-15), serum (13), urine (1, 14), saliva (12) and microsomal incubations (16). HPLC-MS methods offer better sensitivity in comparison with HPLC-UV methods. However, the HPLC-MS equipment is costly and HPLC-UV methods are thus more commonly used in clinical practice.

Unfortunately, the HPLC-UV methods are lengthy because of evaporation and reconstitution steps (6, 7) as well as because of longer chromatographic run (1, 2, 5, 9) compared to the proposed method. Furthermore, it has shown better sensi-tivity and wider method range for MPA analysis than the methods described earlier (4, 8, 10). Hence, the proposed method has some clear advantages in routine applications compared to the above methods.

Aim

The aim of the present study was to perform a validation of the proposed method abiding by the US Food and Drug Administration (FDA) and the International Conference on Harmonisation (ICH) guidelines (17, 18) and also to apply the method in the analysis of MPA in human plasma samples from renal transplant recipients.

Materials and Methods

Drugs and reagents

Mycophenolic acid (MPA, Figure 1b) as solid standard compound (Sigma-Aldrich, Steinheim, Germany) was kindly provided by the representative office of the pharmaceutical company Novartis (Belgrade, Serbia). Internal standard propylparaben (IS, Figure 1c) as solid standard compound was purchased from Sigma-Aldrich (Steinheim, Germany).

Acetonitrile gradient grade for liquid chromatography was purchased from Merck (Darmstadt, Germany). Sodium hydroxide (Merck, Darmstadt, Germany) and ortho-phosphoric acid (Lach-ner, Neratovice, Czech Republic) were of the grade suitable for HPLC.

Apparatus and materials

HPLC analysis was carried out using the chromatographic system Agilent Technologies

1200 (Wilmington, DE, USA) equipped with on-line degasser, binary pump, column oven and photo diode array detector. 10 μL of sample was injected with Agilent 1200 Series High Performan-ce Autosampler G1367B. The water for chromatography was obtained from the purification system Smart 2 Pure (TKA, Niederelbert, Germany). Before use, the mobile phase was degassed and purified by vacuum filtration through 0.45 μm regenerated cellulose membrane filters (Agilent, Böblingen, Germany). The compounds were separated on C18 Bakerbond-BDC analytical column (250 mm x 4.6 mm i.d., particle size 5 μm) (Avantor Performance Materials, Deventer, The Netherlands). The data were collected using the Agilent's ChemStation software. Statistical analysis was carried out using the Microsoft Excel software.

Chromatographic conditions

The mobile phase was 50:50 (v/v) acetonitrile–10 mM ortho-phosphoric acid (pH equal to 2.50 was adjusted with 1 M sodium hydroxide). The flow rate was 1.0 mL/min and column temperature was set at 30°C. Detection was performed at 215 nm.

Human plasma samples

All investigations were approved by the Ethical committee (Faculty of Medicine, University of Niš, Serbia). Healthy volunteers and patients gave their written informed consent. Blank plasma was obtained from ten different healthy volunteers. Plasma samples from renal transplant recipients were obtained from the Clinic of Nephrology (Clinical Centre Niš, Niš, Serbia). Whole blood samples were collected into vacutainers containing EDTA, and separated by centrifugation at 3000 g for 15 minutes. All plasma samples were stored at -20°C until analyis.

Standard solutions, calibration standards and quality control samples

Two standard stock solutions of MPA and IS were prepared in acetonitrile at concentration of 10 mg/mL. Two standard working solutions of MPA and IS contained 1 mg/mL in acetonitrile. One standard working solution of MPA contained 10 μg/mL in acetonitrile. Standard stock solutions were stored at -20°C and standard working solutions were stored at 4-8°C for 1 month. There were no stability related problems.

Each analyte was added separately for the preparation of seven standard curve samples (calibration standards). Four 4 μL and 12 μL of standard working solution of MPA (10 μg/mL) as well as 2 μL, 5 μL, 10 μL, 19 μL and 20 μL of standard working solution of MPA (1 mg/mL) were transfered to seven Eppendorf tubes containing 200 μL of blank human plasma. Ten μL of standard working solution of IS (1 mg/mL) were added to each of the Eppendorf tubes. The concentrations of MPA in calibration standards were 0.2 μg/mL, 0.6 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, 95 μg/mL and 100 μg/mL. The concentration of IS was 50 μg/mL. Zero plasma samples were prepared by adding IS in drug-free plasma to furnish the concentration of 50 μg/mL. Sample preparation was explained in detail in the subsection Sample preparation procedure.

The quality control (QC) plasma samples were prepared to the final concentrations of 0.6 μg/mL (low QC), 50 μg/mL (medium QC) and 95 μg/mL (high QC) of MPA. The concentration of IS was 50 μg/mL. QC (medium) samples were used for the optimization of sample preparation procedure.

QC samples were prepared daily, and parts of all low and high QC samples were stored at -20°C to study the stability after freeze-thaw cycles, as well as the long term stability.

Sample preparation procedure

A 200 μL aliquot of patient plasma was transferred into a 1.0 mL Eppendorf tube, followed by 10 μL of 1 mg/mL IS in acetonitrile and 490 μL of 0.3% trifluoroacetic acid in acetonitrile (v/v). Each tube was capped, vortex mixed for 1 min, and then centrifuged for 10 min at 10000 rpm and temperature of 4ºC. The supernatant was transferred into a clean auto-sampler vial and 10 μL was injected into the HPLC system for analysis.

Results

Method validation

The new HPLC method combined with protein precipitation was subject to validation in accordance with the FDA (17) and ICH (18) guidelines. The following validation characteristics were taken into account: selectivity, linearity, sensitivity, precision, accuracy, absolute recovery and stability.

In order to test the selectivity of the method, freshly prepared spiked samples at lower limit of quantification (LLOQ) level of MPA were compared to blank plasma obtained from 10 healthy volunteers. Coelution was not observed at the retention times of MPA and IS. Figure 2a displays the representative chromatogram of blank plasma sample.

A calibration curve was generated after the analysis of seven calibration standards. For the investigated concentration range of MPA (0.2-100 μg/mL), the following calibration curve was obtained:

$$y = 0.0495x - 0.0249;\ r^2 = 0.9995 \quad (1)$$

where y is the peak area ratio, x is the concentration of the compound and r is the correlation coefficient. The intercept of the calibration curve was tested using the Student t-test. The following results were found for standard deviation of the

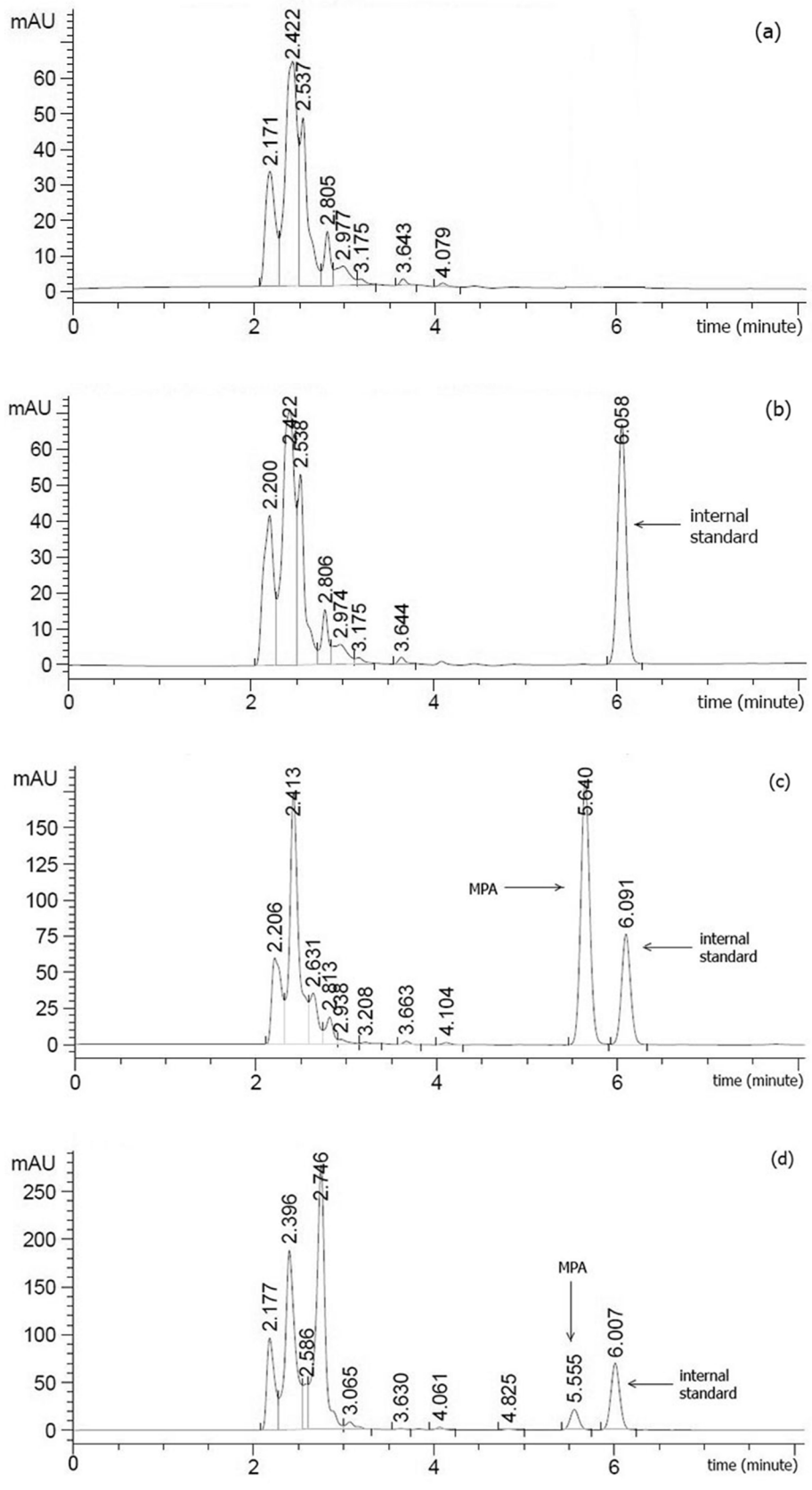

Figure 2. Representative chromatograms of blank plasma (**a**), blank plasma spiked with 50 μg/mL of internal standard (**b**), blank plasma spiked with 50 μg/mL of MPA and 50 μg/mL of internal standard (**c**), and patient's plasma sample (concentration of MPA was 5.62 μg/mL) at 11.0 h after receiving an oral dose of 720 mg MPA (**d**)

slope (Sa), standard deviation of the intercept (Sb) and confidence factor (t_a): Sa = 0.0007, Sb = 0.0403 and t_a = 0.6174. The calculated t_a value was compared to tabular t_a value (p = 0.05 and t_{tab} = 2.37) and therefore the difference of the intercept from zero was found to be insignificant. Figure 2b displays the representative chromatogram obtained from plasma sample spiked with IS. Figure 2c displays the representative chromatogram obtained from plasma sample spiked with MPA and IS.

The limit of detection (LOD) and lower limit of quantification (LLOQ) values for MPA were found to be 0.025 μg/mL and 0.2 μg/mL, respectively. In order to evaluate the accuracy and precision, five replicates at LLOQ level were performed. The accuracy was reported as recovery (R, %) and precision as relative standard deviation (RSD, %). The assessed values are displayed in Table 1.

Intra-day and inter-day accuracy and precision were investigated at QC concentration levels

Table 1. Intra-day precision and accuracy at LLOQ, low QC, medium QC, and high QC concentrations in plasma samples for MPA (*n* = 5)

		Nominal concentration in plasma (μg/mL)			
		0.2	0.6	50	95
MPA					
	Precision (RSD, %)	8.64	7.74	2.88	8.08
	Accuracy (R, %)	107.67	89.31	102.53	96.83
	Found conc. (μg/mL)	0.21	0.54	51.26	91.99

Table 2. Inter-day precision and accuracy at low QC, medium QC, and high QC concentrations in plasma samples for MPA (n = 5)

		Nominal concentration in plasma (μg/mL)		
		0.6	50	95
MPA				
	Precision (RSD, %)	7.86	2.53	4.73
	Accuracy (R, %)	92.66	101.43	96.23
	Found conc. (μg/mL)	0.56	50.71	91.42

Table 3. Absolute recoveries of MPA from plasma samples (*n* = 5)

		Nominal concentration in plasma (μg/mL)		
		0.6	50	95
MPA				
	Recovery (%)	102.30	87.52	102.41
	RSD (%)	4.56	2.54	4.36
	Found conc. (μg/mL)	0.61	43.76	97.29

Table 4. Results of short-term stability test at low QC and high QC concentrations in plasma samples (*n* = 5)

		Nominal concentration in plasma (μg/mL)	
		0.6	95
MPA			
	Recovery (%)	96.24	106.96
	RSD (%)	6.36	4.11
	Found conc. (μg/mL)	0.58	101.61

Table 5. Results of post-preparative stability test at low QC and high QC concentrations in plasma samples (*n* = 5)

		Nominal concentration in plasma (μg/mL)	
		0.6	95
MPA			
	Recovery (%)	96.34	91.07
	RSD (%)	9.49	5.86
	Found conc. (μg/mL)	0.58	86.52

Table 6. Results of long-term stability test at low QC and high QC concentrations in plasma samples (*n* = 5)

		Nominal concentration in plasma (μg/mL)	
		0.6	95
MPA			
	Recovery (%)	91.04	103.40
	RSD (%)	9.84	7.71
	Found conc. (μg/mL)	0.55	98.23

Table 7. Results of freeze-thaw stability test at low QC and high QC concentrations in plasma samples (*n* = 5)

		Nominal concentration in plasma (μg/mL)	
		0.6	95
MPA			
	Recovery (%)	91.52	106.83
	RSD (%)	7.95	4.11
	Found conc. (μg/mL)	0.55	101.49

(low QC, medium QC and high QC). Accuracy was reported as recovery (R, %) and precision as relative standard deviation (RSD, %). The assessed values are displayed in Table 1 and Table 2.

In order to evaluate the efficiency of the sample preparation procedure, the absolute recovery values were calculated. Spiked drug-free plasma samples at low QC, medium QC and high QC levels were subjected to the sample preparation procedure (explained in detail in the subsection Sample preparation procedure). These samples were compared to blank plasma, which had been extracted following the same procedure and then spiked at the same concentration levels. The absolute recovery values and the estimated

Table 8. Results of the assay of plasma samples obtained from renal transplant recipients under treatment with MPA or MMF (n = 5)

Patient	Hours from the last dosage	MPA[1*] or MMF[2*] dosage (mg per day)	Mean concentration[3*] ± SD[4*] (μg/mL)
J. D.	11	1440[1*]	6.08±0.05
S. R.	11	1440[1*]	5.01±0.04
M. T.	11	1440[1*]	1.74±0.02
B. D.	11	1440[1*]	1.48±0.02
L. D.	11	1500[2*]	1.66±0.02
D. I.	11	1500[2*]	12.32±0.11
G.D.S.	11	1440[1*]	5.62±0.05
A. L.	11	720[1*]	1.31±0.02
S. S.	11	1000[2*]	5.96±0.06
R. G.	11	1000[2*]	1.13±0.01
K. S.	11	1500[2*]	3.31±0.03
N. M.	11	1000[2*]	2.00±0.02
S. M.	11	1000[2*]	5.17±0.05
D. A.	11	1000[2*]	2.57±0.03
Z. D.	11	1000[2*]	3.07±0.03
G. S.	11	1000[2*]	3.38±0.04
G.M.S.	11	1000[2*]	0.99±0.01
M. D.	11	1000[2*]	3.33±0.03
P. S.	11	1500[2*]	6.51±0.07
M. I.	11	2000[2*]	2.67±0.03

[1*] Mycophenolic acid (MPA)
[2*] Mycophenolate mofetil (MMF)
[3*] Mean concentration of MPA in plasma (μg/mL)
[4*] Standard deviation (μg/mL)

concentrations from human plasma for MPA are displayed in Table 3.

The following stability tests were performed at low QC and high QC levels of MPA: short-term stability, post-preparative stability, long-term stability and freeze-thaw stability. The results of the above-mentioned tests are displayed in Table 4-7.

Clinical application

The results of the assayed plasma samples, obtained from twenty renal transplant recipients under treatment with MPA or MMF, are displayed in Table 8. The representative chromatogram obtained from a patient's plasma sample is displayed in Figure 2d. The patients were given not only MPA but also the following drugs: acetylsalicylic acid, amlodipine, bisoprolol, bromazepam, carvedilol, cilazapril, cyclosporin, enalapril, isosorbide mononitrate, lamivudine, methyldopa, metoprolol, nifedipine, omeprazole, pantoprazole, prednisone, prednisolone, ranitidine, sulfamethoxazole, tacrolimus, tamsulosin, tenofovir, and trimethoprim. For these 20 plasma samples, coelution was not observed at the retention times of MPA and IS. In order to additionally examine the selectivity of the method, plasma samples were assayed from healthy volunteers who took the above-mentioned drugs and coelution was not noticed at the retention times of MPA and IS.

Discussion

The proposed method is selective since coelution was not observed at the retention times of MPA and IS from freshly prepared spiked samples at LLOQ level compared to blank plasma obtained from 10 healthy volunteers.

The calibration curve showed good linearity over the investigated range for MPA (0.2-100 μg/mL), since r^2 was equal to 0.9995. Moreover, Student t-test proved that the difference of the intercept from zero was not significant.

The proposed method has a good sensitivity, because LOD and LLOQ values for MPA were 0.025 μg/mL and 0.2 μg/mL, respectively.

After performing the investigation of intra-day and inter-day accuracy and precision, it was noticed that RSD (%) and recovery (R, %) values were in accordance with the FDA guidelines (17) (precision of 20% and accuracy of 80-120% at LLOQ level; precision of 15% and accuracy of 85-115% at low QC, medium QC and high QC levels). The obtained RSD (%) and recovery (R, %) values are displayed in Table 1 and Table 2.

The absolute recovery values of MPA showed an appropriate efficiency of the sample preparation procedure. Moreover, they appeared to be independent of concentration. The absolute recovery values and the estimated concentrations from human plasma for MPA are displayed in Table 3.

After performing the stability tests at low QC and high QC levels (short-term stability, post-preparative stability, long-term stability and freeze-thaw stability), RSD (%) values for MPA were below 9.84%, while recovery (%) ranged from 91.04% to 106.96%. In conclusion, an appropriate stability of MPA was proven in all investigations. The results of the above-mentioned tests are displayed in Table 4-7.

Plasma samples obtained from twenty renal transplant recipients under treatment with MPA or MMF were assayed. The results are displayed in Table 8. No interference was observed, even though these patients were concomitantly treated with the following drugs: acetylsalicylic acid, amlodipine, bisoprolol, bromazepam, carvedilol, cilazapril, cyclosporin, enalapril, isosorbide mononitrate, lamivudine, methyldopa, metoprolol, nifedipine, omeprazole, pantoprazole, prednisone, prednisolone, ranitidine, sulfamethoxazole, tacrolimus, tamsulosin, tenofovir and trimethoprim. Moreover, plasma samples were assayed from healthy volunteers who were given the above-mentioned drugs and no interference was noticed.

On balance, the proposed method can be used in the routine analysis of MPA in plasma samples obtained from renal transplant recipients on polytherapy. It allows therapeutic drug monitoring as well as pharmacokinetic studies due to adequate sensitivity.

Conclusion

The new HPLC method combined with protein precipitation has been validated for the analysis of MPA in human plasma from renal transplant recipients. The intra-day and inter-day precision and accuracy of the method were good, with RSD values in the range from 2.53% to 8.64 % and recovery (R, %) values from 89.31% to 107.67%. The method showed appropriate linearity for MPA in the range 0.2 - 100 μg/mL with correlation coefficient equal to 0.9995. The efficiency of the sample preparation procedure was confirmed by calculating absolute recovery values to be from 87.52% to 102.41%. No interferences were observed during the analysis of patients' plasma. A wide method range, good sensitivity and short chromatographic run are all in favor of the method regarding its possible clinical practice utilization. In conclusion, the proposed method is suitable for routine MPA analysis in the plasma obtained from renal transplant recipients on polytherapy. It can be used not only for therapeutic drug monitoring, but also for pharmacokinetic studies.

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VALIDACIJA HPLC METODE ZA ODREĐIVANJE MIKOFENOLNE KISELINE U HUMANOJ PLAZMI NAKON IZVRŠENE TRANSPLANTACIJE BUBREGA

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Jednostavna, brza i osetljiva HPLC metoda, kombinovana sa precipitacijom proteina, razvijena je i validirana u cilju određivanja mikofenolne kiseline u humanoj plazmi nakon izvršene transplantacije bubrega. Radi izvođenja analize, korišćena je C18 Bakerbond-BDC analitička kolona (250 mm x 4,6 mm; 5 μm). Optimalni uslovi za hromatografsko razdvajanje su mobilna faza acetonitril - 10 mM fosfatni pufer, pH 2,5 (50:50, v/v), protok od 1,0 mL/min, temperatura 30°C i detekcija na 215 nm. Ukupno trajanje hromatografskog rana iznosi oko 6 minuta. Precipitacija proteina plazme izvedena je korišćenjem 0,3% trifluorosirćetne kiseline u acetonitrilu (v/v). HPLC metoda, kombinovana sa precipitacijom proteina, podvrgnuta je validaciji. Linearnost je potvrđena u koncentracionom opsegu 0,2-100 μg/mL za mikofenolnu kiselinu, sa vrednošću korelacionog koeficijenta od 0,9995. Štaviše, preciznost metode u toku jednog i u toku više dana je dobra, sa relativnom standardnom devijacijom nižom od 8,64%, dok tačnost metode obuhvata vrednosti u opsegu od 89,31% do 107,67% za mikofenolnu kiselinu. Na kraju, metoda je uspešno primenjena u cilju analiziranja uzoraka plazme bolesnika sa transplantiranim bubregom na politerapiji. *Acta Medica Medianae 2016;55(4):28-36.*

Ključne reči: *mikofenolna kiselina, humana plazma, precipitacija proteina, visokoefikasna tečna hromatografija*