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Application of experimental design in optimization of solid phase extraction of mycophenolic acid and mycophenolic acid glucuronide from human urine and plasma and SPE-RP-HPLC method validation

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Abstract

The aim of this study was to develop and optimize a solid phase extraction (SPE) procedure for purification of mycophenolic acid (MPA) and its metabolite mycophenolic acid glucuronide (MPAG) in biological samples. During optimization process chemometric approach was applied. First, in screening experiments fractional factorial design (FFD) was used for selecting the variables which affected the extraction procedure. The ionic strength of the phosphate buffer in the washing step and the percentage of acetonitrile in the elution step were statistically significant for the recovery of MPAG while the percentage of acetonitrile and pH of the washing solution were statistically significant for that of MPA. Afterwards, the significant variables were optimized using central composite design (CCD). The developed SPE method included phosphate buffer (pH 2.4; 0.056 M) in the washing step, and the mixture of acetonitrile and phosphate buffer of which pH was adjusted to 2.4 (70:30, v/v) in the elution step. The investigation was applied to both urine and plasma and the nature of biological matrix appeared to be of no importance. The extraction from both matrixes showed good repeatability with relative standard deviations up to 6% for MPAG and 8% for MPA, and recovery around 100% for both substances. Furthermore, new SPE-RP-HPLC method for determination of MPA and MPAG in both humane urine and plasma has been validated. The great advantage of this method is the chromatographic run of only 3 min.

Keywords: Solid phase extraction; Fractional factorial design; Central composite design; Mycophenolic acid; Mycophenolic acid glucuronide; Method validation

1. Introduction

The introduction of mycophenolate mofetil (MMF) (Fig. 1a), a powerful non-nephrotoxic, non-cardiotoxic immunosuppressant with less bone marrow toxicity, significantly improved the immunosuppressive therapy, especially among patients with impaired graft function [1]. MMF is the current primary immunosuppressant for the prevention of renal allograft rejection. It is a pro-drug and actually its active metabolite mycophenolic acid (MPA) (Fig. 1b) is an immunosuppressive agent which inhibits lymphocyte proliferation by blocking the *de novo* synthesis pathway of guanoxine nucleotides. MPA is primarily metabolized by glucuronidation at the phenolic hydroxyl group to mycophenolic acid glucuronide (MPAG) (Fig. 1c), which is the major urinary excretion product of the drug. MPAG is pharmacologically inactive although it may be hydrolyzed *in vivo* to form free MPA [2].

Thus, the pharmacokinetic studies of MPA and MPAG from organ transplant patients are of crucial importance. Complexity and variability of the biomatrix are the main problems in the development of bioanalytical methods. Having this in mind, the process of purification and concentration of biomatrix samples is the most important step in the development of new bioanalytical methods. For this reason, the aim of the study was to determine and optimize all significant variables of the solid phase extraction (SPE) procedure for urine and plasma samples. During method optimization and development the chemometric approach was applied. With limited number of experiments the combination of the variables which provides the best analytical response was obtained. In the chemometric approach each parameter can be examined and optimized in a predefined range by conducting a series of

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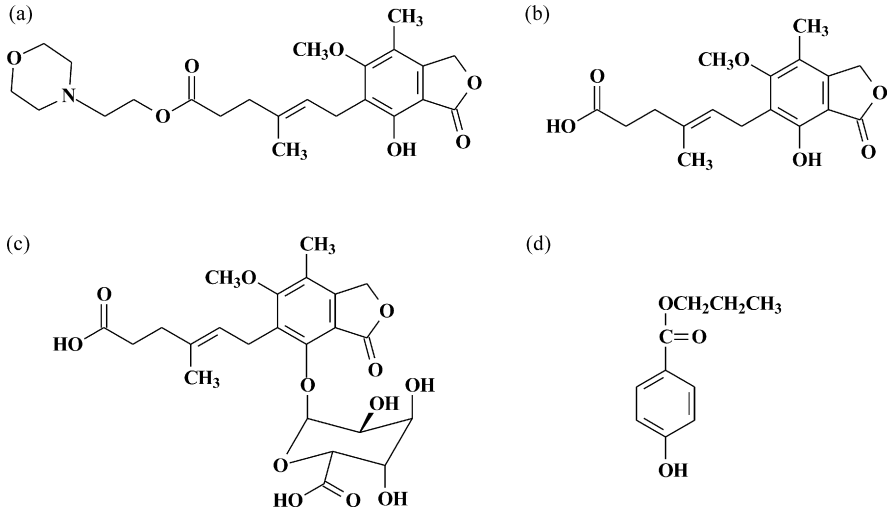

Fig. 1. Structures of mycophenolate mofetil (a) mycophenolic acid (b), mycophenolic acid glucuronide (c) and prophyl parabenum (d).

experiments in which the values for several parameters are changed at the same time. Two major groups of experimental design are important: screening and optimization designs [3]. In the case of detailed modelling it is often desirable at a first stage to reduce the number of factors via screening designs to a smaller number of main factors that are to be studied in detail (employing optimization designs) for which both squared and interaction terms in the model are of interest [4].

In the published papers, several methods have been found covering purification of biological samples by precipitation of plasma proteins with organic solvents [5–8], ultrafiltration [9,10] and molecularly imprinted SPE [2]. Among reported papers, SPE methods have also been presented [11–15]. However, to our knowledge, neither papers based on optimization and development of the extraction procedure nor chemometric approach was applied. Furthermore, until now, there are only papers which take into the consideration clean-up process of plasma, but not urine samples. The purification of urine samples and concentration of the investigated substances in urine are very important at patients with renal disorders, including patients after kidney transplantation. So, besides plasma, the influence of urine matrix on repeatability and recovery of SPE procedure was included in the investigation.

After the optimization of SPE procedure, a new SPE-RP-HPLC method has been validated. All previously published methods for simultaneous determination of MPA and MPAG from biological matrixes have as a practical inconvenience the use of gradient elution, ion pair reagents and long chromatographic runs. Differences in hydrophilic properties of MPA (relatively non-polar, $\log P = 3.88 \pm 0.38$ for uncharged form) and MPAG (relatively polar, $\log P = 0.49 \pm 0.52$ for uncharged form) make the simultaneous determination of both components in one analytical run a difficult task [15]. But, this new, isocratic SPE-RP-HPLC method enabled us to determine both substances from both matrixes in a single run of only 3 min.

2. Experimental

2.1. Chemicals and reagents

MPA and internal standard propyl parabene (I.S.) (Fig. 1d) were purchased from Sigma (Taufkirchen, Germany). MPAG was kindly supplied by Roche (Palo Alto, California).

Reagent grade 85% orthophosphoric acid and potassium dihydrogen phosphate were obtained from Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germany), respectively. Acetonitrile HPLC-grade was obtained from Lab Scan (Dublin, Ireland) and purified water from a Simplicity 185 purification system, Millipore (Billerica, Massachusetts, USA) was used in the preparation of buffer solutions.

2.2. Instrumentation and materials

HPLC analysis was performed with an Agilent Technologies (Palo Alto, CA, USA) HP 1100 chromatograph equipped with HP 1100 binary pump, HP 1100 UV–visible detector and Rheodyne 20 μl loop injector. Data was acquired with ChemStation software from HP. Compounds were separated on Chromolith RP-18e (100 mm × 4.6 mm, macropore size 2 μm, mesopore size 13 nm) column (Merck, Darmstadt, Germany). Before use, the mobile phase was degassed and vacuum filtered through 0.45 μm nylon membranes (Alltech Associates, Loceren, Belgium).

The clean-up procedure consisted of solid phase extraction and was performed using BAKER spe-12G vacuum manifold system from J.T. Baker (Deventer, Holland) coupled to a vacuum pump from KNF Neuberger (Freiburg, Germany).

For extraction procedure, BakerBond SPE columns were used; column size 1 ml and filled with 100 mg silica-boned C18, purchased from J.T. Baker (Deventer, Holland).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile-potassium dihydrogen phosphate buffer (pH 2.4; 0.040 M) (28:72, v/v). The pH of the water phase was adjusted to 2.4 with 85% orthophosphoric acid. The column was thermostatted at $25 \pm 0.2\,^{\circ}C$, flow rate was 5 ml/min and detection was performed at 215 nm.

2.4. Urine and plasma samples

Drug free human urine and plasma samples were obtained from ten healthy volunteers. Plasma and urine samples from patients were obtained from Children's University Hospital in Belgrade. Blood samples were collected into tubes containing 4% water solution of sodium citrate and separated by centrifugation at $3000 \times g$ for 10 min. All samples were stored at $-80\,^{\circ}C$ until analysis.

2.5. Preparation of stock and working solutions

Stock solutions of MPA, MPAG and propyl parabene as internal standard were prepared in acetonitrile–water (50:50, v/v) from standard substances in the concentration of 10, 35 and 5 mg/ml, respectively.

Then, working solutions of 25, 75, 500, 2500, 5000, 10,000, 12,500 μg/ml of MPAG, 25, 75, 125, 250, 500, 1000, 1250 μg/ml of MPA and 1250 μg/ml of I.S. were prepared in acetonitrile–potassium dihydrogen phosphate buffer (pH 4.0; 0.04 M) (15:85, v/v). These working solutions were used for spiking blank humane urine.

The working solutions for spiking blank humane plasma were prepared in concentration of 5, 15, 25, 50, 200, 250, 350 μg/ml of MPAG, 5, 15, 50, 100, 200, 250, 300 μg/ml of MPA and 250 μg/ml of I.S. These solutions were also prepared in acetonitrile–potassium dihydrogen phosphate buffer (pH 4.0; 0.04 M) (15:85, v/v).

All solutions were stored at the refrigerator temperature (4–8 °C) for 1 month. The stability of stock and working solutions was not investigated for a longer period of time.

2.6. Calibration standards

The volume of 100 μl aliquots of the above mentioned working solutions was transferred to the glass vials containing 0.5 ml blank human urine and plasma. After SPE procedure which is thoroughly described in Section 2.8, samples contained: 1, 3, 20, 100, 200, 400, 500 μg/ml of MPAG and 1, 3, 5, 10, 20, 40, 50 μg/ml of MPA in urine and 1, 3, 5, 10, 40, 50, 70 μg/ml of MPAG and 1, 3, 10, 20, 40, 50, 60 μg/ml of MPA in plasma samples. All samples finally contained 50 μg/ml of I.S. The zero urine and plasma samples were prepared in the same way by adding only internal standard in drug free humane urine and plasma to yield the final concentration of 50 μg/ml.

2.7. Quality control samples

For the validation of the assay, three pools of quality control (QC) samples were prepared. QC urine samples were prepared in concentrations of 3 (low), 200 (medium), 400 (high) μg/ml of MPAG and 3 (low), 10 (medium), 40 (high) μg/ml of MPA. QC plasma samples were prepared to the final concentrations of 3 (low), 10 (medium) and 50 (high) μg/ml of both MPAG and MPA. In all QC samples the concentration of I.S. was 50 μg/ml. The preparation of QC samples was also preformed in the described way.

QC samples were prepared daily and parts of the three pools of all QC samples were stored at $-20\,^{\circ}C$ to study the stability after thaw-freeze cycles in long term.

The samples, which were used for the optimization of extraction procedure, were prepared in the same concentration as QC (medium) samples.

2.8. Extraction procedure of urine and plasma samples

An aliquot of 0.5 ml of KH_2PO_4 (pH 2.4; 0.040 M) was added to all prepared samples, which was followed by brief vortex mixing. The SPE cartridges were conditioned with 2 ml of methanol, followed by 2 ml of phosphate buffer (pH 2.4; 0.040 M). The prepared plasma and urine samples were then applied to the cartridges manually and washed with 2 ml of phosphate buffer (pH 2.4; 0.056 M). Then, the substances were eluted with 0.5 ml of acetonitrile–phosphate buffer (pH 2.4; 0.040 M) (70:30, v/v) for both urine and plasma samples.

After the extraction procedure, 2.0 ml of the phosphate buffer (pH 2.4; 0.040 M) was added to the urine samples. The dilution was necessary because of much higher concentrations of investigated substances in urine.

2.9. Experimental design

Fractional factorial design (FFD) was employed as a screening design. In this way, the number of experiments was kept low based on the assumption that interaction effects between three or more parameters are small compared to main and two-variable interaction effects. Thus, it is possible to select a fraction of the full factorial design and omit several combinations of parameters from experimental plan [3]. The number of experiments in FFD is given by $2^{k-p} + C$, where k is the number of variables, C the number of replicates at center point and p the whole number that indicates how fractionated the experimental design will be. When p is zero, the experimental design is full. In our study the FFD involved eight experiments, carried out randomly and additional four experiments were repeated at the central point in order to estimate the experimental error and the significance of each variable.

Afterwards, from the results of the FFD, a central composite design (CCD) was built using the same variables as in the FFD, but excluding those which lacked significance. The CCD was built from the full factorial 2^k to which a star design was added. The length of the arms of the star determined the number of levels and the shape of the experimental design. The CCD was

completed by addition of a center point. The total number N of experiments with k factors is: $N = 2^k + 2k + c$. The first term is related to the full factorial design, the second to the star points and the third to the center point. The length of the arms of the star (α) played a major role for the appearance of the CCD. If $\alpha \neq 1$, each variable will assume five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) [16]. In order to maintain the highest symmetry as possible, $-\alpha$ and $+\alpha$ levels were located at $-(2^{1/2})$ and $+(2^{1/2})$, respectively. Also, as it is very important to estimate pure experimental uncertainty, central point was repeated four times [17]. All other experiments were performed randomly and without replication.

Finally, the response surface plots were presented in order of visualization and rapid selection of optimal conditions. Response surface plots are presented in three-dimensional space and clearly show the influence of two factors on the recovery value in the investigated as well as slightly outside of the investigated region [18].

The optimization and success of the extraction procedure was evaluated by calculating absolute recovery values. For the estimation of absolute recovery, the internal standard was added after the SPE procedure of the investigated substances. During the optimization process the blank plasma and urine samples were analyzed before each experiment in FFD and CCD in order to exclude the possibility of any endogenous interference.

For statistical analysis, construction of the experimental matrix and evaluation of the results, Excel 2003 (version 11) included in Microsoft Office 2003 was used. The analysis of variance (ANOVA) and response surface plots were achieved with StatSoft Statistica 5.0 Software 1997 edition.

3. Results and discussion

3.1. Optimization of the solid phase extraction variables

In the first stage of our investigation several experiments were conducted in order to decide which variables should be included in the screening design.

Reversed-phase sorbents (C18 in our study) are commonly used in SPE when aqueous samples are involved. The mechanism of interaction is mainly van der Waals' forces (although, occasionally second interactions such as hydrogen bonding and dipole–dipole interactions occur) and all analytes should be mainly non-ionized to be in appropriate form for bonding on the solid phase [19]. MPAG is stronger acid compared to MPA (pK_a (MPAG) = 2.8 and pK_a (MPA) = 4.5), and therefore more ionized at pH above 3.0. Hence, it seems that the pH value of the plasma and urine samples applied to SPE sorbents should be lower than pK_a. Nevertheless, the influence of the pH of the biological samples was investigated conducting a few experiments. The samples whose pH was adjusted from 2.4 to 4.0 gave the highest recoveries. On the other hand, adjustment of pH $\geq$ 5 resulted at higher percentage of ionized molecules of MPAG and MPA and significant losses in the extraction procedure were observed. Since cleaner extracts were obtained from urine and plasma samples with pH adjusted at higher values, it was decided to adjust pH of all samples to 4.0 prior to SPE. In addition, on the basis of the chemical properties of the investigated substances, our first and second variables to be investigated were the pH of the washing solution and pH of the elution solution. All chosen variables and their investigated levels in 2^{4-1} FFD are presented in Table 1.

Table 1
Investigated variables and their levels studied in the FFD 2^{4-1} design

Variables	Investigated levels		
	−1	0	+1
(*A*) Ionic strength of the phosphate buffer (KH_2PO_4) in washing step (mM/l)	8	28	48
(*B*) pH value of the phosphate buffer (KH_2PO_4) in the washing step	2.4	4.7	7.0
(*C*) pH value of the phosphate buffer (KH_2PO_4) in the elution step	2.4	4.7	7.0
(*D*) Acetonitrile in solution in the elution step (%)	40	60	80

Phosphate buffer had to be added to plasma and urine samples for two reasons. Firstly, plasma samples should be diluted 1:1 prior to SPE procedure because plasma is very viscous. Secondly, both matrixes needed the pH adjustment as previously explained [19]. Considering this, the addition of the phosphate buffer to both urine and plasma samples before the extraction procedure was necessary. The 0.040 M phosphate buffer was chosen because it is the component of the mobile phase. The pH of the phosphate buffer must be 2.4 in order to adjust the pH of the samples at 4.0 prior to SPE procedure. On the other hand, the necessity of the addition of phosphate buffer as well as its concentration in the washing solution was the third variable chosen to be investigated in the FFD.

The last, fourth variable was the percentage of acetonitrile in the elution step. The percentage of acetonitrile is very important for quantitative extraction and degradation of van der Waals' forces. Phosphate buffer (0.040 M KH_2PO_4) was used in the elution solution as the aqueous phase since the addition of phosphate buffer was essential for peak shape of MPAG.

As previously mentioned, the importance of all investigated variables was estimated using 2^{4-1} FFD. The FFD plan of the experiments and the obtained results expressed as absolute recovery for MPA and MPAG in urine samples are presented in Table 2. According to the obtained recovery values, the estimated effects and then the standardized effects were calculated. The next step in our investigation was to estimate the importance of the variables. Critical t-value, for $\alpha = 0.05$ and 3 degrees of freedom (d.f.), was 3.182 for both substances. All factors whose absolute values of the standardized effects are above critical t-value are statistically significant and the ones below this value are statistically insignificant. Pareto charts, of which the length of the bars is proportional to the absolute value of the standardized effects, are presented in Fig. 2. The dashed line represents critical t-value and the importance of the presented variables can be easily noticed.

According to the obtained results, the percentage of acetonitrile had important impact on extraction of both MPA and MPAG.

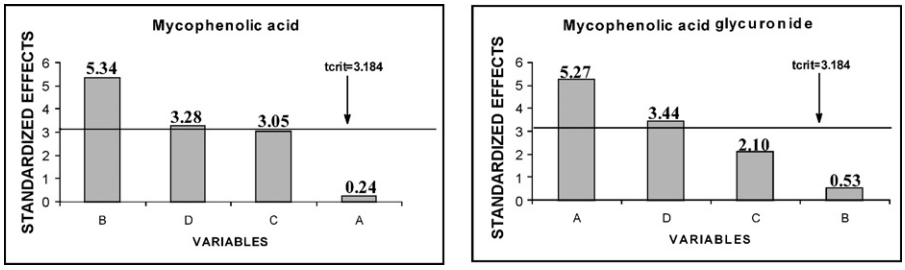

Fig. 2. Pareto charts of standardized effects (absolute value) obtained from the FFD for MPA and MPAG, where examined variables are: (*A*) ionic strength of the washing solution (KH_2PO_4, mM/l), (*B*) pH value of the washing solution (KH_2PO_4), (*C*) pH value of the elution solution and (*D*) percentage of acetonitrile in the elution solution.

Table 2
The plan of the experiments for the FFD 2^{4-1} design for four variables with the fourfold repetition of the central point and corresponding recovery values for MPA and MPAG from urine samples

Exp. no.	Variables				Recovery values	
	A	*B*	*C*	*D*	MPA	MPAG
1	−1	−1	−1	−1	85.0	97.9
2	+1	−1	−1	+1	99.4	100.0
3	+1	+1	−1	−1	42.4	82.1
4	+1	−1	+1	−1	98.5	92.9
5	−1	−1	+1	+1	84.6	48.8
6	+1	+1	+1	+1	88.7	79.3
7	−1	+1	−1	+1	75.0	74.3
8	−1	+1	+1	−1	91.9	90
9	0	0	0	0	63.0	63.6
10	0	0	0	0	68.7	64.6
11	0	0	0	0	68.7	66.5
12	0	0	0	0	67.5	66.4

A, ionic strength of the washing solution (KH_2PO_4, mM/l). *B*, pH value of the washing solution (KH_2PO_4). *C*, pH value of the elution solution. *D*, percentage of acetonitrile in the elution solution.

Table 4
The plan of the experiments for CCD design for two variables with the fourfold repetition of the center point and corresponding recovery values for MPA and MPAG from urine samples

Exp. no.	Variables		Recovery values, MPA	Variables		Recovery values, MPAG
	B	*D*		*A*	*D*	
1	+1	+1	90.3	+1	+1	68.5
2	+1	−1	16.0	+1	−1	11.7
3	−1	+1	93.1	−1	+1	104.6
4	−1	−1	88.2	−1	−1	91.4
5	0	$+2^{1/2}$	93.2	0	$+2^{1/2}$	33.3
6	0	$-2^{1/2}$	1.2	0	$-2^{1/2}$	92.8
7	$+2^{1/2}$	0	91.7	$+2^{1/2}$	0	92.9
8	$-2^{1/2}$	0	91.3	$-2^{1/2}$	0	85.6
9	0	0	89.3	0	0	99.1
10	0	0	89.9	0	0	101.9
11	0	0	90.3	0	0	97
12	0	0	86	0	0	98

A, ionic strength of the washing solution (KH_2PO_4, mM/l). *B*, pH value of the washing solution (KH_2PO_4). *D*, percentage of acetonitrile in the elution solution.

Considering other variables, the ionic strength was significant in the washing step for the extraction of MPAG, and pH value of the washing solution on the extraction of MPA.

In further work these variables were thoroughly studied employing CCD. The investigated variables, their domains and obtained optimal values are presented in Table 3. The plan of experiments for CCD and the obtained recovery values are presented in Table 4 and finally, the Pareto charts obtained from CCD are presented in Fig. 3. The estimation of the importance of the factors was performed in the same way as in FFD.

All investigated variables in CCD showed to have significant influence on the extraction procedure of the investigated substances. For MPAG the percentage of acetonitrile, as well as its interaction with the concentration of phosphate buffer used in the washing step showed to have significant influence on the extraction procedure. Considering MPA, the percentage of acetonitrile, the pH value of the washing solution and their mutual interactions showed to have a great impact on the recovery values.

Based on the results of the performed experiments polynomial equations were obtained by multiple linear regression analysis (MLR) in the following form:

$$y = 75.2 - 9.3x_1 + 26.2x_2 + 17.4x_1x_2 + 2.0x_1^2 - 20.3x_2^2 \text{ for MPA} \quad \text{and} y = 52.3 - 13.2x_1 - 1.7x_2 + 10.9x_1x_2 - 6.7x_1^2 - 19.8x_2^2 \text{ for MPAG}$$

Table 3
Investigated variables and their levels studied in the CCD design and obtained optimal values for MPA (considering variables *B* and *D*) and MPAG (considering variables *A* and *D*)

Variables	Investigated levels					Obtained optimal values
	$-\alpha$	−1	0	+1	$+\alpha$	
(*A*) Ionic strength of the phosphate buffer (KH_2PO_4) in washing step (mM/l)	0	8	28	48	56	56
(*B*) pH value of the phosphate buffer (KH_2PO_4) in the washing step	1.5	2.4	4.7	7.0	7.9	2.4
(*D*) acetonitrile in solution in the elution step (%)	32	40	60	80	88	70

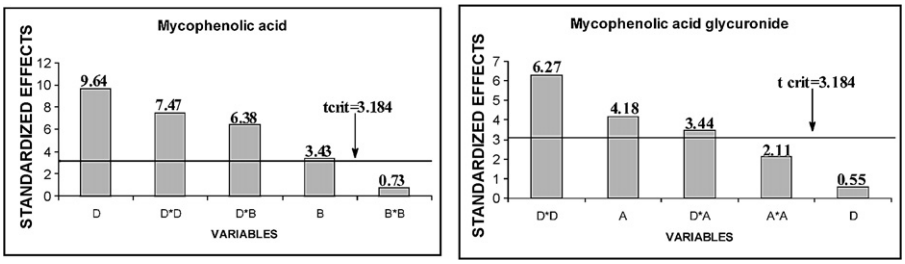

Fig. 3. Pareto charts of standardized effects (absolute value) obtained from the CCD for MPA and MPAG, where examined variables are: (*A*) ionic strength of the washing solution (KH_2PO_4, mM/l), (*B*) pH value of the washing solution (KH_2PO_4) and (*D*) percentage of acetonitrile in the elution solution.

Table 5
Calibration curve parameters for MPA and MPAG in plasma and urine samples

Compound	Matrix	$y = ax + b$	r	S_a	S_b	t_α
MPA	Plasma	$0.0508x + 0.0939$	0.9991	0.0157	0.4349	0.2159
	Urine	$0.1577x - 0.0302$	0.9998	0.0013	0.0331	0.9131
MPAG	Plasma	$0.0361x + 0.0012$	0.9999	0.0001	0.0048	0.2543
	Urine	$0.0643x - 0.2577$	0.9997	0.0012	0.3719	0.6929

S_a, S_b, standard deviations of the slope and intercept. t_α, calculated deviation value for intercept. $t_{0.05}$, 2.447 tabular value obtained from Student's *t*-test.

In these equations, y is the measured response (recovery) associated with each factor level combination, x_1 is the pH value of the washing solution in equation for MPA and the concentration of phosphate buffer in the washing solution for MPAG and x_2 is the percentage of acetonitrile in the elution step for both substances.

In addition to this, for better visualization and rapid choose of true optimum conditions surface plots were constructed. The representative plots for both substances are presented in Fig. 4 in which optimal conditions, the interactions between variables and their mutual dependence can be clearly noticed. Mutual interactions between variables, acetonitrile and pH value of solution in washing step, are statistically significant and could not be neglected. The efficiency of the extraction for MPA depends on the percentage of acetonitrile but it is highly changeable according to the pH of the washing solution (Fig. 4A). If a higher percentage of acetonitrile is used, the impact of the pH of the washing solution on the recovery value is also greater. As it could be noticed, the greater recovery values were obtained with the use of higher percentage of acetonitrile and lower pH of the washing solution at the same time. Relying on the response surface plots, the best conditions for extraction of MPA are pH of 2.4 for the washing solution and 70% of acetonitrile. Moreover, due to the higher polarity of MPAG, higher recoveries were obtained with lower percentage of acetonitrile if at the same time the concentration of phosphate buffer in the washing step was greater than 0.03 M (Fig. 4B). The use of the phosphate buffer in the concentration range from 0 to 0.03 M has negative influence on the extraction efficiency but with the concentrations above 0.03 M this influence becomes positive. It must be noted that the influence of the concentration of the phosphate buffer is much greater if the percentage of acetonitrile in the elution step is lower. As it could be seen, the greatest recovery values were obtained with 50% of acetonitrile and with 0.056 M phosphate buffer in the washing step.

Considering all obtained results and the fact that with 70% of acetonitrile in elution step recovery of MPAG is still above 90%, the satisfactory conditions have been chosen and are thoroughly described in Section 2.8.

As the last step of the optimization of the method, the validation of the obtained model was done by the analysis of variance. The results for the ANOVA are briefly summarized here to

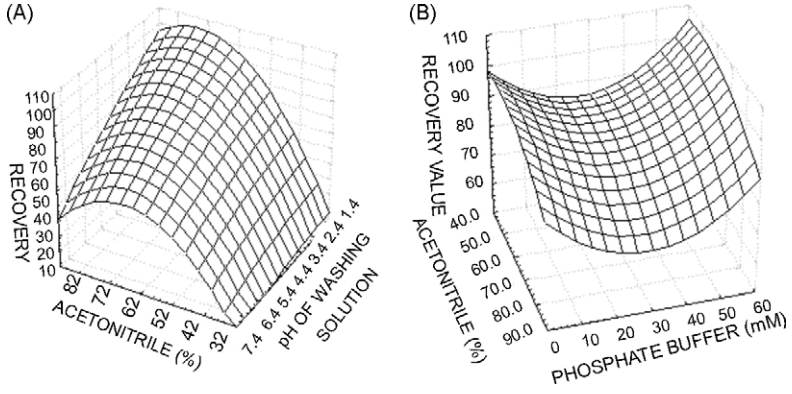

Fig. 4. Response surface plots for MPA (A) and MPAG (B).

Table 6
Intra-day precision and accuracy at LLOQ, low, medium and high concentrations in spiked urine and plasma samples for MPA and MPAG ($n = 5$)

	Nominal concentration (μg/ml)							
	Plasma				Urine			
	1	3	10	50	1	3	10	40
MPA								
Precision (R.S.D., %)	3.46	0.55	2.78	0.66	1.28	1.60	1.05	0.08
Accuracy (*R*, %)	91.46	101.41	107.36	109.44	115.34	95.80	101.37	95.87
Found concentration (μg/ml)	0.91	3.04	10.74	54.72	1.15	2.87	10.14	38.35
	Nominal concentration (μg/ml)							
	Plasma				Urine			
	1	3	10	50	1	3	200	400
MPAG								
Precision (R.S.D., %)	6.78	5.04	4.00	1.31	3.07	3.49	0.31	4.39
Accuracy (*R*, %)	96.71	101.55	100.33	103.24	90.22	87.87	111.52	101.35
Found concentration (μg/ml)	0.97	3.05	10.03	51.62	0.90	2.64	223.04	405.4

n, number of replicates in the same concentration.

show the absence of lack of fit and confidence of the developed model. The regression lack of fit is determined performing an *F*-test by comparing SSlof/SSpe (the lack of fit and pure error sum of squares, SSlof and SSpe, respectively) with the tabled *F* value for appropriate degrees of freedom at the desirable confidence level. In our case, tabled $F_{1,5}$ at 95% confidence level was 230.20. This value was compared with calculated *F* value which was 46.01 for MPA and 4.22 for MPAG. As the calculated quotients are lower then the tabled value, there is no model lack of fit and the model can be accepted for both substances.

All experiments in 2^{4-1} FFD and CCD were also performed with plasma samples. Influence of the matrix was not noticed and the same model explains the extraction procedure from the plasma samples as well.

3.2. Method validation

The new SPE-RP-HPLC method for determination of MPA and MPAG in humane urine and plasma was validated following the guidelines of the International Conference on Harmonisation (ICH) and according to the industry consensus on bioanalytical validation, reflected in Food and Drug Administration (FDA) guidelines [20,21].

3.2.1. Selectivity

To insure the selectivity of the method the blank urine and plasma obtained from 10 volunteers (different age and sex) were chromatographically screened for interfering substances. No co-elution was observed at the retention times of MPA, MPAG and internal standard comparing with freshly prepared spiked samples at LLOQ level. The corresponding chromatograms of blank

Table 7
Inter-day precision and accuracy at low, medium and high concentrations in spiked urine and plasma samples for MPA and MPAG ($n = 5$)

	Nominal concentration (μg/ml)					
	Plasma			Urine		
	3	10	50	3	10	40
MPA						
Precision (R.S.D., %)	7.20	3.16	2.42	1.80	2.05	2.03
Accuracy (*R*, %)	98.17	106.46	106.06	97.52	103.55	98.17
Found concentration (μg/ml)	2.94	10.65	53.03	2.92	10.35	39.27
	Nominal concentration (μg/ml)					
	Plasma			Urine		
	3	10	50	3	200	400
MPAG						
Precision (R.S.D., %)	6.79	4.23	1.76	4.50	2.21	6.39
Accuracy (*R*, %)	98.28	100.50	101.12	89.90	108.32	105.80
Found concentration (μg/ml)	2.95	1.01	50.56	2.70	216.64	423.2

n, number of replicates in the same concentration.

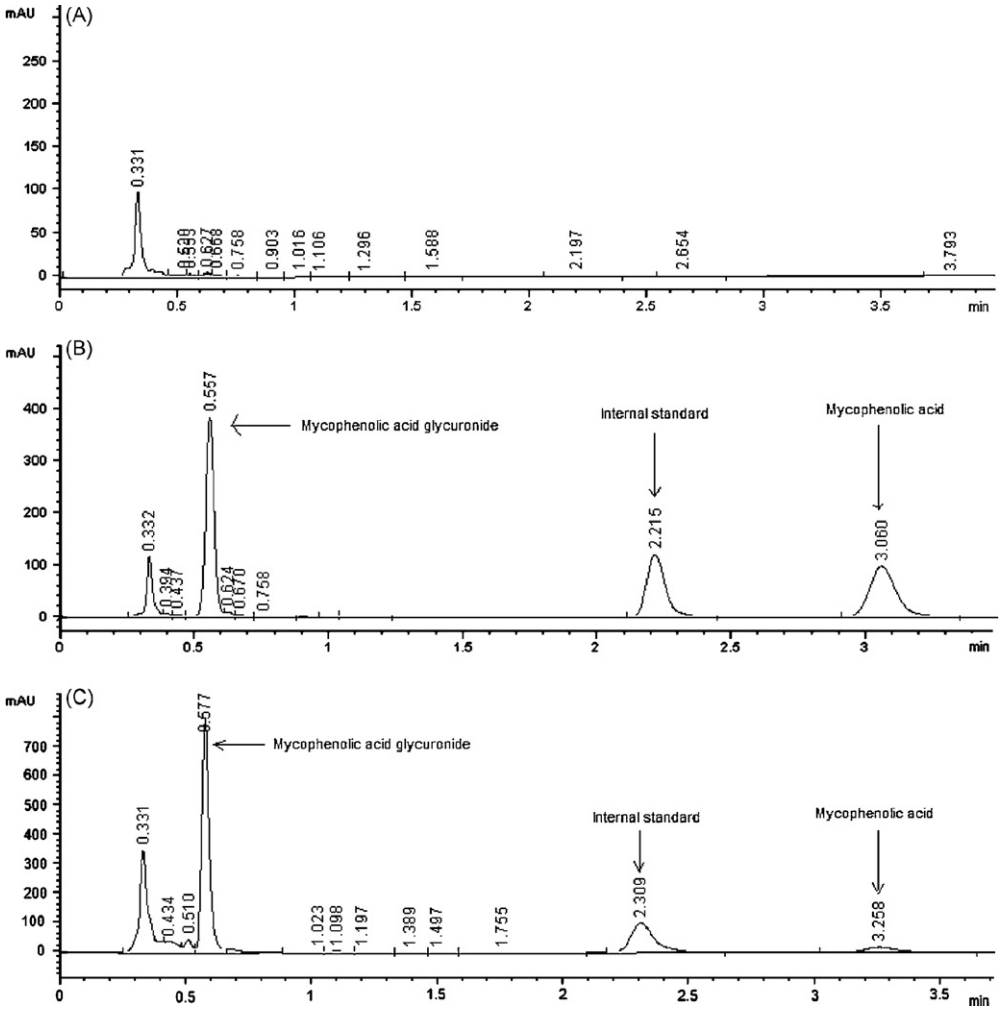

Fig. 5. The representative chromatograms of blank plasma (A), plasma samples spiked with 10 μg/ml of MPA, 20 μg/ml of MPAG and 50 μg/ml of internal standard (B), and plasma sample (3.15 μg/ml of MPA and 62.5 μg/ml of MPAG) of stable kidney transplant patient at 30 min after receiving an oral dose of 500 mg of MMF (C).

plasma and urine samples are presented in Fig. 5A and Fig. 6A.

3.2.2. Limit of detection (LOD) and lower limit of quantification (LLOQ)

LOD for MPA and MPAG was found to be 0.1 and 0.2 μg/ml, respectively, in both plasma and urine. When this method is applied to urine and plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies.

The lowest standard on the calibration curve should be accepted as the limit of quantification if analyte response at the LLOQ should be at least 5 times the response compared to blank response and analyte peak should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% [20]. 1 μg/ml for MPA and MPAG both in plasma and urine, showed to meet these criteria.

The accuracy and precision were evaluated by five solutions per substance. Accuracy is reported as recovery (*R*, %), precision as relative standard deviation (R.S.D., %) and assessed values are presented in Table 6.

3.2.3. Linearity

A sufficient number of standards should be used to adequately define the relationship between the corrected area (ratio analyte area (MPA and MPAG)/IS area) and concentration. Concentrations of standards were chosen on the basis of the concentration range expected in a patient's plasma and urine samples. Calibration curve consisted of blank sample (urine and plasma), a zero sample (urine and plasma spiked with internal standard) and seven non-zero samples of each substance covering the expected range, including LLOQ [20]. The calibration curve was obtained applying linear regression model based on the least square method. The concentration range was: 1–50 μg/ml for MPA and 1–500 μg/ml for MPAG in urine and 1–60 μg/ml for MPA and 1–70 μg/ml for MPAG in plasma matrix. The concentration of internal standard in all samples was 50 μg/ml. Linearity was assessed based on parameters reported in Table 5. Each calibration concentration was assayed in triplicate. The corresponding chromatograms obtained from plasma and urine samples spiked with standard solutions are presented in Fig. 5B and Fig. 6B, respectively.

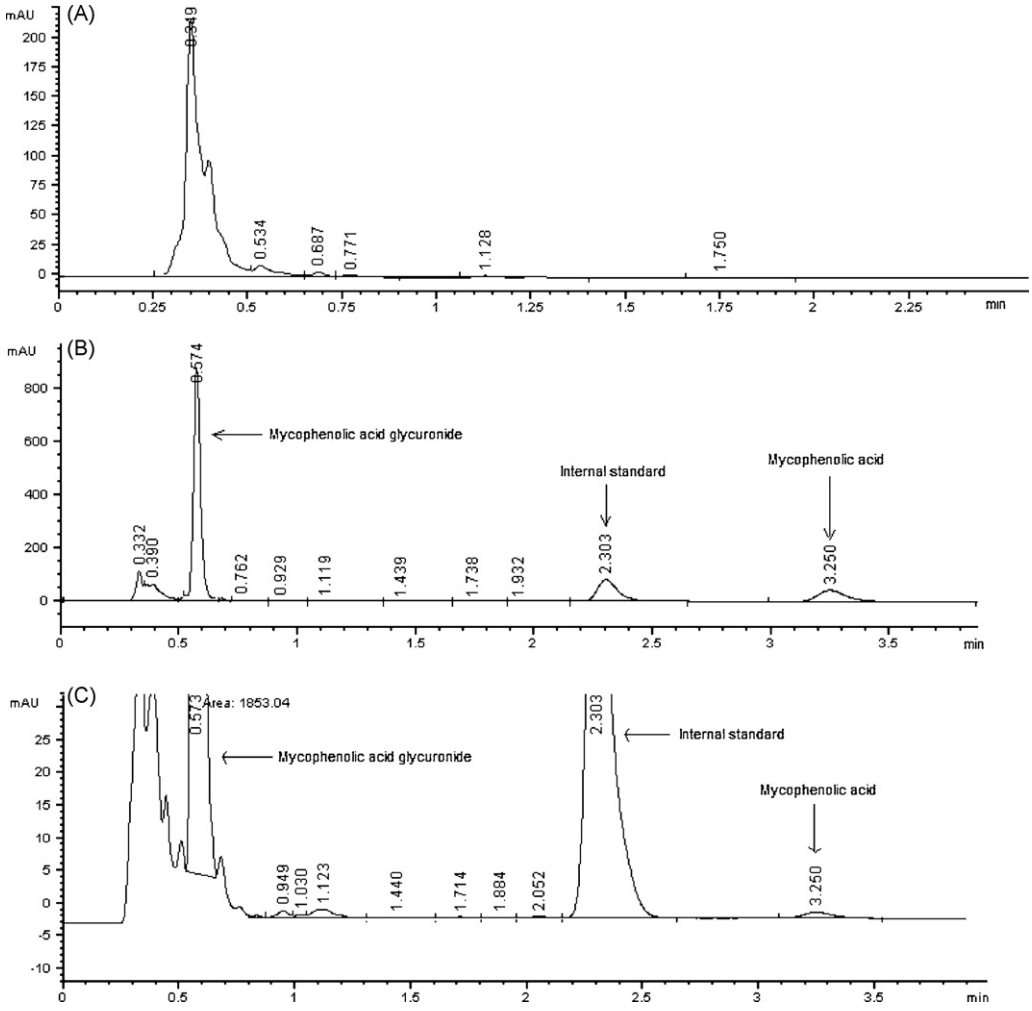

Fig. 6. The representative chromatograms of blank urine (A), urine samples spiked with 5 μg/ml of MPA, 50 μg/ml of MPAG and 50 μg/ml of internal standard (B), and urine sample (1.8 μg/ml of MPA and 54.85 μg/ml of MPAG) of stable kidney transplant patient at 4.0 h after receiving an oral dose of 500 mg of MMF (C).

3.2.4. Precision and accuracy

Accuracy and precision were determined by replicate analysis of samples containing known amounts of the analyte. They were measured using five determinations per concentration. Precision and accuracy were determined within the same day (intra-day precision and accuracy) and over 3 consecutive days (inter-day precision and accuracy).

The precision of the assay was evaluated by means of the R.S.D. for each concentration of MPA and MPAG. The request by FDA guidelines for intra-day and inter-day precision is that R.S.D. (%) does not exceed 15%, except for the LLOQ where it should not exceed 20%. The accuracy of the assay was reported as *R* (%) of MPA and MPAG at each concentration. The limits in the FDA guidelines are the same as above. The results are presented in Tables 6 and 7.

3.2.5. Recovery from urine and plasma samples

To evaluate the efficiency of the extraction procedure the absolute recovery of SPE procedure was calculated from five replicates of plasma and urine samples spiked with working solutions to achieve three different concentration levels. These samples were compared with blank plasma and urine samples which had been extracted following the same SPE procedure but spiked just before the injection. In all cases the internal standard was added after the SPE procedure (to achieve the final concentration of 50 μg/ml).

The absolute recovery values as well as the estimated concentrations from human plasma and urine for both MPA and MPAG are presented in Table 8. The recovery did not appear to be concentration dependent and it could be concluded that the nature of biological matrix showed no influence.

3.2.6. Stability

The stability of the compounds was evaluated at two different concentrations, each being assayed five times. First, the investigation evaluated the stability at room temperature for 6 h. During this period biological samples were stored at room temperature and then extracted and analyzed. In this way the stability was assured in the period of collection and handling. The period of 24 h was also evaluated to confirm the stability of the samples if pharmacokinetic study requires storing biological samples in autosampler at room temperature. To evaluate this kind of stability, samples were extracted and stored for 24 h at room temperature prior to analysis. Then, the stability was

Table 8
Absolute recoveries of MPA and MPAG from plasma and urine samples ($n = 5$)

	Nominal concentration (μg/ml)					
	Plasma			Urine		
	3	10	50	3	10	40
MPA						
Recovery (%)	98.2	100	101	99.2	97.5	96.4
R.S.D. (%)	7.20	3.16	2.42	5.20	4.42	4.01
Found concentration (μg/ml)	2.95	10	50.5	2.98	9.75	38.56
	Nominal concentration (μg/ml)					
	Plasma			Urine		
	3	10	50	3	200	400
MPAG						
Recovery (%)	92.4	92.3	88.1	92.5	90.5	88.4
R.S.D. (%)	5.04	4.30	1.31	4.36	2.01	3.12
Found concentration (μg/ml)	2.77	9.23	44.05	2.78	181	353.6

n, number of replications in the same concentration.

studied after three thaw-freeze cycles and at −20 °C stored for 3 months for urine samples and 6 months for plasma samples. The main values and standard deviations of the ratios between the concentration found and the initial concentration were used for the stability evaluation. MPA showed outstanding stability in both matrixes with recovery value from 95% to 105% in all investigated conditions. The stability of MPAG was also satisfactory with the lowest recovery of 88.9% (R.S.D. 4.4%) after the 3rd month (long term stability) of storing urine samples at −20 °C. In all other experimental conditions the recovery value was above 91.9%. It could be concluded at the end that substances showed to be stable in the investigated experimental conditions.

3.2.7. Clinical application

The proposed method was subsequently applied to plasma and urine samples obtained from stabile patients under immunosuppressive treatment with Mycophenolate mofetil after kidney transplantation. Corresponding chromatograms obtained from a patient's plasma and urine samples are presented in Fig. 5C and Fig. 6C, respectively. Along with Mycophenolate mofetil, patients were co-administrated with methylprednisolone, nifedipine, carvedilol, tramadol, sulfamethoxazole and trimethoprim, ranitidine, miconazole, and furosemide. In all cases, no co-elution was observed at the analytes or internal standard retention times. But, as we used nonselective UV detection further investigation of possible co-elution was required. In order to insure the selectivity of the method we investigated plasma and urine samples of the volunteers who were administrated with above-mentioned drugs except Mycophenolate mofetil and no interferences were observed. In this way the applicability of the method was confirmed. Anyway, if it is possible it is always advisable to take plasma and urine samples from patients before starting the immunosuppressive therapy to evaluate the suitability of the method in each patient, due to the high interindividual variability and the intake of other drugs simultaneously.

4. Conclusion

The chemometric approach in this study, fractional factorial and central composite design, allowed us to establish the optimal conditions for solid phase extraction of MPA and MPAG from biological matrixes with limited number of experiments. The method showed to be suitable for purification of plasma and urine samples. No influence of the investigated biological matrixes on the extraction procedure was observed. Furthermore, a sensitive, accurate, precise and reproducible SPE-RP-HPLC method for determination of MPA and MPAG in urine and plasma has been established and validated without interferences from endogenous compounds and co-administered drugs. The short chromatographic run time of only 3 min is advantageous in the routine practice.

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