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Multicriteria optimization methodology in development of HPLC separation of mycophenolic acid and mycophenolic acid glucuronide in human urine and plasma

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ABSTRACT

Multicriteria optimization methodology was applied for development of isocratic reversed-phased HPLC method for simultaneous determination of mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG) in human urine and plasma. In the first stage of method development, pH value of the water phase, percentage of acetonitrile, temperature of the column and flow rate of the mobile phase were investigated using fractional factorial design.

Afterwards, the optimal conditions were found employing central composite design and Derringer's desirability function. Two goals were considered, the retention factor of the MPAG to be in the range, between 0.8 and 1.118 which allowed well separation of MPAG from the urine and plasma peaks, and the shortest possible total analysis run time. Then, the obtained sigmoid functions were used to transform the optimization criteria into the desirability values.

The satisfactory chromatographic conditions were obtained with mobile phase consisted of acetonitrile–phosphate buffer (pH 2.4; 0.04 M KH_2PO_4) (28:72, v/v). The separation was performed on C_{18} Chromolith column (100 mm × 4.6 mm) with flow rate of 5 mL/min, the temperature of the column was 25 °C and the chosen wavelength for simultaneous determination of MPA and MPAG was 215 nm. The MPAG eluted at 0.552 min and the duration of run was 3.092 min.

Afterwards, the method was subjected to validation. Linearity was observed over the concentration range of 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 method showed intra-day and inter-day precision with relative standard deviation lower then 5% and accuracy as recovery (%) between 100 ± 5%.

1. Introduction

Mycophenolate mofetil (MMF, Fig. 1a) is an immunosuppressive agent which is mostly used in solid organ transplant patients to prevent organ rejection [1]. MMF is pro drug which is rapidly absorbed and hydrolyzed to form free mycophenolic acid (MPA) (Fig. 1b), the active metabolite. 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 [2]. MPA is further metabolized through glucuronidation to form an inactive metabolite mycophenolic acid glucuronide (MPAG) (Fig. 1c) which exists in plasma in up to 100-fold greater concentrations than MPA [3,4]. A second and less abundant metabolite is the acyl glucuronide (AcMPAG). Unlike MPAG, AcMPAG is pharmacologically active [4].

The monitoring of MPA levels is necessary, in particular during the induction and adaptation periods when multiple factors may affect MPA pharmacokinetics: impaired renal function, drug–drug interactions between MPA and cyclosporine, glucocorticoids, antacids, and metronidazol [5]. The literature suggests the necessity of measuring AUC, because a single time point does not correlate well with AUC, the target level of MPA AUC_{0-9} should be approximately 30 ng h/mL to avoid organ failure and toxicity [6,7]. On the other hand, MPAG is inactive metabolite extensively bound to serum albumin, from which it can displace MPA, and is excreted in both urine and bile. Excretion of MPAG into bile allows enteric organisms with glucuronidase activity to cleave MPAG back to MPA which can undergo enterohepatic recirculation. Accordingly, two MPA peaks in plasma are usually seen: one following the

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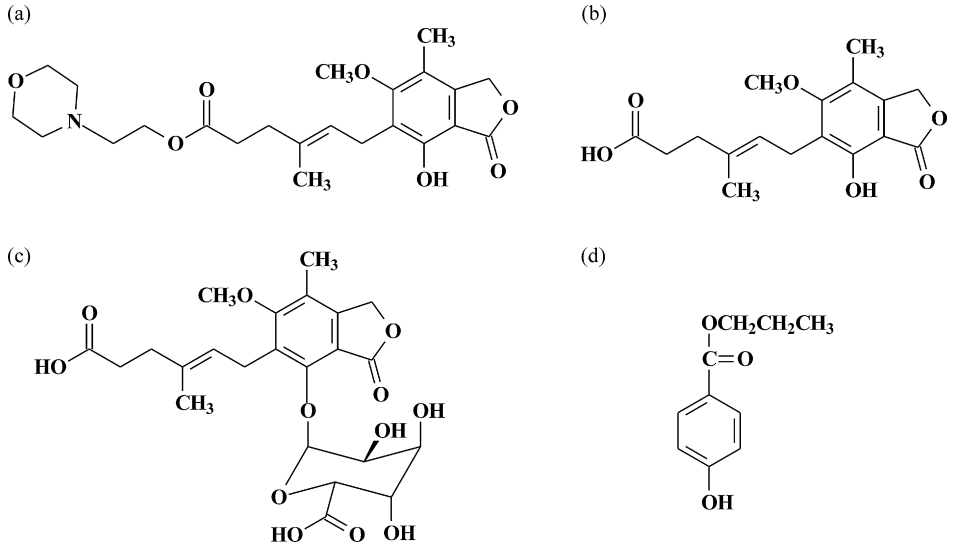

Fig. 1. Structures of mycophenolate mofetil (a), mycophenolic acid (b), mycophenolic acid glycuronide (c) and prophyl parabenum (d).

first adsorption and occurs 0.5–1 h after oral administration and second and smaller peak representing adsorption from the distal bowel following enterohepatic recirculation 4–6 h after oral intake of the parent drug Cellcept [7]. Because of these facts, measuring the concentration of MPAG in urine and plasma samples can be useful. Such research requires fast analytical methods due to the high number of samples that should be processed. Therefore the aim of our study was to develop simple, sensitive, precise, accurate and above all rapid method for analysis of MPA and MPAG in both human urine and plasma.

The optimization process is one of the most important and complex stages during method development. The most popular approach is "one variable at a time". This is a time consuming methodology which requires a high number of experiments and above all it cannot provide information about possible interactions of the investigated factors. Based on these facts, the classical methodology may be inefficient in determining the true optimal conditions [8]. Because of this, the alternative approach was chosen and included fractional factorial design and response surface methodology (RSM) (in our case central composite design, CCD). Thus, RSM makes it possible to obtain a map of the response in the form of a surface and to predict the considered responses over the whole domain. Moreover, the obtained models can be used for optimization when multiple responses are considered [9]. In our study, the multicriteria decision making approach, Derringer's desirability function, was used for the evaluation of two different chromatographic performance goals. The first goal was to obtain the retention factor of MPAG between 0.8 and 1.12, as it allowed well separation of MPAG from the urine and plasma peaks, and the second goal was to provide the shortest possible total analysis run time.

Considering methods for simultaneous determination of MPA and MPAG, several methods have been reported. The early reported methods rely on indirect measurement of MPAG after enzymatic hydrolysis to MPA [10]. Another practical inconvenience of reported methods was the use of ion pair reagents [11–17] which are regarded as extremely column damaging, and gradient elution methods [2,18–20] as a time consuming procedures. Isocratic methods without use of ion pair reagents and gradient elution have been also established. But, the lower sensitivity [21] and time consuming chromatographic run [4,21–25] were the disadvantages of these methods. Some of the mentioned methods [4,23,25] use the protein precipitation for sample purification which could be useful as time saving procedure. On the other hand, unlike SPE methods, protein precipitation approach does not allow the possibility of concentrating the biological samples. This could be a problem, especially when working with large number of samples with high variability, like biological samples. The LC/MS methods for quantification of investigated substances have also been established [26–29]. Although these methods have obvious advantages, still, few clinical laboratories are in position to perform LC/MS methods. Therefore new sensitive, precise, accurate and rapid RP-HPLC UV method for drug monitoring of MPA and MPAG in pediatric patients is more then welcome in this area. Among the mentioned LC/MS papers, LC/UV method has also been developed [29]. But this method has serious drawbacks concerning the gradient elution and total analysis run time of 18 min. In the latest published papers the AcMPAG has also been determined [4,23,25,27,29]. But to our knowledge, not only that this is the most rapid method but also there are no reported papers based on optimization and development of the chromatographic procedure. Applying multicriteria optimization approach enabled us to investigate and determine complete chromatographic behavior of the investigated substances. This could be valuable for further research and establishment of other chromatographic methods.

At the end, the developed method was subjected to validation according to FDA [30] and ICH [31] guidelines and the proposed method met all validation criteria.

2. Experimental

2.1. Chemicals and reagents

MPA and internal standard prophyl parabenum (I.S.) (Fig. 1d) were purchased from Sigma (Taufkirchen, Germany). MPAG was kindly supplied by Roche (Palo Alto, CA). 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, MA, 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, Lokeren, Belgium).

The clean-up procedure of plasma samples consisted of a 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, Baker Bond SPE columns were used; column size 1 mL and filled with 100 mg silica-boned C_{18}, 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 thermostated at 25 ± 0.2 °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 × *g* for 10 min. All samples were stored at −80 °C until analysis.

2.5. Preparation of stock and working solutions

Stock solutions of MPA, MPAG and prophyl parabenum (I.S.) 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, 10000 12500 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 human urine. The working solutions for spiking blank human plasma were prepared in concentrations 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 refrigerator temperature (4–8 °C) for one 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 into glass vials containing 0.5 mL of 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 I.S. in drug free 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 μg/mL (low), 200 μg/mL (medium), 400 μg/mL (high) of MPAG and 3 μg/mL (low), 10 μg/mL (medium), 40 μg/mL (high) of MPA. QC plasma samples were prepared to the final concentrations of 3 μg/mL (low), 10 μg/mL (medium) and 50 μg/mL (high) 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 previously described way. QC samples were prepared daily and parts of the three pools of all QC samples were stored at −20 °C to study the stability after thaw–freeze cycles in long term. The samples, which were used for the optimization of chromatographic 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 and this 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 samples were then applied to the cartridges manually and washed with 2 mL of phosphate buffer (pH 2.4; 0.056 M). Afterwards, the substances were eluted with 0.5 mL of acetonitrile–phosphate buffer (pH 2.4; 0.040 M) (70:30, v/v) in case of both urine and plasma samples. After the extraction procedure, 2.0 mL of the phosphate buffer (pH 2.4; 0.040 M) was added in urine samples. The dilution was necessary because of much higher concentrations of investigated substances in urine.

3. Results and discussion

Differences in hydrophilic properties of MPA (relatively nonpolar, $\log P = 3.88 \pm 0.38$ 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 [7]. The retention factor of MPAG should be greater than 0.8 since this would insure separation of MPAG from urine and plasma peaks. At the same time, this could influence and prolong the total analysis run time. As the second aim of the optimization was to achieve the shortest possible analysis run time, this response was also necessary to take into consideration. When several responses are evaluated through an experimental design, it is unlikely that the optimum points reached individually for each factor coincide in all the cases. Faced with this situation, it was necessary to look for a compromise zone where all the experimental responses fulfill the specifications imposed by the researcher to achieve the aims proposed [32]. In order to optimize retention factor of MPAG and total analysis run time, the combination of experimental design and Derringer's desirability function was found to be necessary.

3.1. Screening design

Preliminary experiments involved the investigation of the variables that might have influence on the behavior of analyzed substances in the chromatographic system. Generally, the HPLC separation depends on the physical and chemical properties of the compounds, composition and pH of the mobile phase, column temperature and stationary phase properties. According to this, in the screening phase all factors that could influence the separation of MPA and MPAG as well as their domains were determined.

During preliminary experiments, nature of the stationary phase was firstly investigated. The C_{18} packing columns showed to be the most suitable according to the nature of the compounds. At one side, XTerra C_{18} column enabled better peak shape of the compounds towards conventional C_{18} columns due to its hybrid technology. On the other side, monolithic packing, as one of the new developments, can achieve a high-speed separation. As the paramount of the modern pharmaceutical analysis is to provide higher column efficiency and shorter analysis time, we decided to continue our investigations on Chromolith C_{18} column. This enabled us to provide satisfactory peak symmetries and to work with flow rates up to 5 mL/min. For that reason, the influence of the flow rates on the chromatographic behavior of the compounds was also included in the screening design.

Afterwards, the percentage of organic modifier was examined. Between different organic modifiers, acetonitrile showed the best characteristics considering peak shape and retention parameters. As the retention time of the compounds was unreasonably prolonged when amount of acetonitrile was below 25%, this percentage was chosen as the low level. At the same time, 35% of organic modifier was chosen as the high level since above this percentage, no separation could be achieved.

Considering peak broadening and symmetries, the addition of phosphate buffer was necessary. Satisfactory peak symmetries were accomplished with 40 mM KH_2PO_4. Since lower concentrations of the phosphate buffer did not give satisfactory results and higher concentrations showed to have no influence on the retention parameters, buffer concentration was held constant and was not taken into the consideration in the further investigation.

The pH values of the water phase were decided to be varied from 2.4 to 4.8. The pK_a values of the investigated compounds (pK_a (MPAG) = 2.8 and pK_a (MPA) = 4.5) showed that MPAG is stronger acid compared to MPA. As MPAG is completely ionized at pH two units above its pK_a, the investigated pH area should be tighter. On the other hand, the lower pH prolonged retention time of MPA and consequently total analysis run time. The idea was to examine wider pH range and investigate the statistical significance of pH variable and all possible interactions between pH and all other variables (depending on the combination of the variables it might be possible to work on higher pH values and still obtain satisfactory retention factor of MPAG) which would lead to the choice of a thorough experimental optimum.

The temperature was examined in the range from 25 to 35 °C. Since the peak symmetries of both compounds were considerably worse on higher temperatures, wider range of temperature was not investigated.

After preliminary experiments, 2^{4-1} fractional factorial design (FFD) was performed. FFD was employed prior to central composite design to reduce the number of variables. In that way only statistically significant variables would be studied in detail employing optimization designs [33].

Applying FFD the number of experiments can be kept low based on the assumption that interaction effects among three or more parameters are small compared to main effects and two-variable interaction effects [34]. The number of experiments in FFD is given by $2^{k-p} + C$, where k is the number of variables, C number of replicates and p a whole number that indicates how fractionated the experimental design will be. When p is zero, the experimental design is full [35]. The repetition of four experiments at central point provided a precise estimate of an experimental error and the significance of each variable. All other experiments were performed randomly and without repetition.

The investigated variables and their domains are presented in Table 1. Only two levels were used so that the variables were considered as discrete values and no continuous second order response model could be estimated. High and low levels of each variable are based on the preliminary investigations and are denoted as +1 and −1. Experimental plan for FFD is reported in Table 2. The observed response was retention factor for both substances and according to obtained values, estimated and then standardized effects were calculated. The obtained values of estimated and absolute values of standardized effects for MPA and MPAG are presented in Table 3. The effect of each variable was then tested using a Student's t-test with a corresponding p-value. The factors in which p-values were above 0.05 were considered as "statistically significant". A graphical display of the standardized effect (the absolute value of the estimated effect divided by its standard error estimate) of each variable was given in a Pareto chart. The length of the bars is proportional to the absolute value of the standardized effects, and is presented in Fig. 2. A line in the Pareto chart indicated the threshold for a test at level $p = 0.05$ (for $t_{crit} = 3.184$) [36,37].

Not only the absolute, but also the standardized effects are presented in the Fig. 2 in order to see weather the influence of the variable on the response is positive or negative. If a standardized effect is a negative value, it indicates that increase of a variable leads to decrease of a retention parameter and if it is positive, the increase of variable leads also to increase of the response.

From the obtained results it could be concluded that percentage of acetonitrile and pH of the water phase showed statistically significant influence on the chromatographic behavior of both substances. Both variables have negative effect on the retention factor of MPA and MPAG. According to the obtained results, only these two variables were further investigated in detail using CCD and multiple response analysis. The temperature of the column and

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

Variables	Investigated levels		
	−1	0	+1
(*A*) pH value of the phosphate buffer (KH_2PO_4)	2.4	3.6	4.8
(*B*) Acetonitrile in the mobile phase (%)	25	30	35
(*C*) Temperature of the column (°C)	25	30	35
(*D*) Flow rate of the mobile phase (mL/min)	1	3	5

Table 2
The plan of the experiments for the FFD 2^{4-1} design for four variables and corresponding retention factors for MPA and MPAG.

Experiment no.	Variables				Retention factors	
	A	*B*	*C*	*D*	MPA	MPAG
1	−1	−1	−1	−1	15.85	1.54
2	+1	−1	−1	+1	11.51	0.33
3	+1	+1	−1	−1	2.66	0.06
4	+1	−1	+1	−1	11.30	0.34
5	−1	−1	+1	+1	12.87	1.24
6	+1	+1	+1	+1	2.75	0.13
7	−1	+1	−1	+1	3.16	0.30
8	−1	+1	+1	−1	3.04	0.30

A – pH value of the phosphate buffer (KH_2PO_4); *B* – acetonitrile in the mobile phase (%); *C* – temperature of the column (°C); *D* – flow rate of the mobile phase (mL/min).

Table 3
Estimated effects and absolute values of standardized effects for MPA and MPAG.

Variables	Estimate effects		Standardized effects (absolute values)	
	MPA	MPAG	MPA	MPAG
A	−0.84	−0.325	5.18	23.52
B	−4.99	−0.33	30.82	24.71
C	−0.40	−0.03	2.49	2.12
D	−0.32	−0.03	1.99	2.33

A – pH value of the phosphate buffer (KH_2PO_4); *B* – acetonitrile in the mobile phase (%); *C* – temperature of the column (°C); *D* – flow rate of the mobile phase (mL/min).

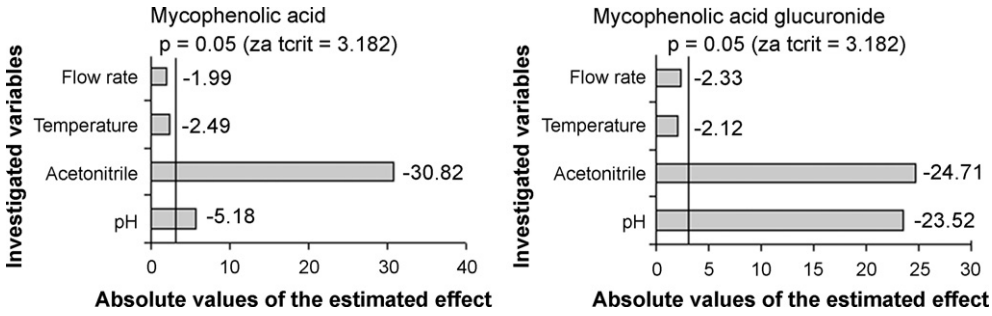

Fig. 2. Representative Pareto charts of standardized effects (absolute value) obtained from the FFD shows the influence of studied variables on the retention factors of MPA (A) and MPAG (B).

flow rate showed to have no statistically significant influence on the chromatographic behavior of the investigated compounds and was further held constant. It was chosen to hold temperature at the 25 °C concerning the best peak symmetries. For flow rate was decided to be 5 mL/min because no significant back pressure was noticed and the speed of the analysis was approved.

3.2. Response surface methodology

Now a detailed optimization procedure was applied in order to find out the exact values of the variables which would fulfill all proposed goals.

The purpose of RSM is to obtain a predictive model for each response which adequately represents changes in the response within the zone of interest, depending on the input variables. Moreover, the obtained models could be used for optimization in the problem of treating multiple responses [9].

In order to carry out a quadratic regression on the model coefficients, each design variable has to be studied at least at three distinct levels, and consequently the central composite design is often used to provide estimation of a second-order equation. Among the standard designs used in RSM, the CCD represents a good choice because of its high efficiency with respect to the number of required runs [38]. Important variables to be tested during the optimization process were pH of the water phase (40 mM KH_2PO_4) and percentage of the acetonitrile in the mobile phase. Generally, The CCD was built from the full factorial 2^k to which a star design was added. 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. If the length of the arms of the star in star design is $\alpha = 1$, the star points lie on the faces of the cube and the experimental domain is the same as previously defined by a fractional factorial design (Table 1). This kind of design is called face cantered cube CCD. Also, as it is very important to estimate pure experimental uncertainty, central point was repeated four times [39,40]. All other experiments were performed randomly and without replication to minimize the effect of uncontrolled variables that may introduce a bias on the measurements. Thus, for two variables a total of 12 experiments were required including four replications in the center point. The plan of experiments for CCD and the measured responses are presented in Table 4.

Based on the results of the performed experiments the following polynomial equations were obtained:

$$y = 2.75 - 0.32x_1 - 1.07x_2 + 0.23x_1x_2 - 0.59x_1^2 + 0.58x_2^2 \quad \text{for MPA,}$$

where y is optimized response (retention time) and

$$y = 0.51 - 0.25x_1 - 0.29x_2 + 0.14x_1x_2 - 0.14x_1^2 + 0.07x_2^2 \quad \text{for MPAG,}$$

where y is optimized response (retention factor).

In both equations, x_1 is the pH value of the water phase and x_2 is the percentage of acetonitrile in the mobile phase.

For the calculated response models, one for each considered response, were evaluated by means of analysis of variance (ANOVA). 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 $F_{3,3}$ at 95% confidence level was 9.28. This value was compared with calculated F value which was 0.0104 for MPA and 0.020 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. It could be concluded that models adequately represent the influence of the investigated variables on the response, retention time of MPA and retention factor of MPAG.

In order to reach a compromise among the responses which could better satisfy the goals, the Derringer's desirability function was used, thus converting a multi-response problem into a single-response one [9]. In these situations where compromise between goals is necessary desirability function becomes an interesting and powerful tool. The Derringer's desirability function, D, is defined as the geometric mean, weighted, or otherwise, of the individual desirability functions. The expression that defines the Derringer's desirability function is:

$$D = [d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \ldots \times d_n^{pn}]^{1/n},$$

where pi is the weight of the response, n the number of responses and d_i is the individual desirability function of each response obtained from the transformation of the individual response of each

Table 4
The plan of the experiments for the CCD design for two variables with the four-fold repetition of the center point and corresponding retention factors for MPA and MPAG further used in the multiple response procedure.

Experiment no.	Variables		Investigated responses	
	A	*B*	MPA Retention time	MPAG Retention factor
1	+1	+1	1.084	0.08
2	−1	+1	1.266	0.299
3	+1	−1	3.854	0.33
4	−1	−1	4.972	1.11
5	+1	0	1.778	0.11
6	−1	0	2.37	0.6
7	0	+1	1.359	0.233
8	0	−1	5.123	0.89
9	0	0	2.8	0.5
10	0	0	2.4	0.54
11	0	0	3	0.525
12	0	0	3	0.53

A – pH value of the phosphate buffer (KH_2PO_4); *B* – acetonitrile in the mobile phase (%).

Table 5
Criteria for the optimization of the individual responses.

Response	Goal	Lower limit	Upper limit
Retention time of MPA	Minimize	0.96	5.04
Retention factor of MPAG	Range	0.8	1.12

experiment. The scale of the individual desirability function ranges between $d_i = 0$, for a completely undesired response, to $d_i = 1$ for a fully desired response. Weights can range from 0.1 to 10. Weights lower than 1 give less emphasis to the goal, whereas weights greater than 1 give more emphasis to the goal (in both cases, d_i varies in a non-linear way while approaching to the desired value). But with a weight of 1, d_i varies in a linear way. In the present study we have chosen weights equal to 1 for all the responses.

For goal maximum, the desirability curve is defined by the equation:

$$d_i = \left[\frac{Y_i - \text{Low}_i}{\text{High}_i - \text{Low}_i}\right]^{\text{wt}_i}$$

$$\text{Low}_i < Y_i < \text{High}_i$$

where Y_i is the predicted response using the fitted model, High_i and Low_i are the highest and the lowest values obtained for the response i, respectively, and wt_i is the weight.

For a goal of minimum, the desirability is defined as equation:

$$d_i = \left[\frac{\text{High}_i - Y_i}{\text{High}_i - \text{Low}_i}\right]^{\text{wt}_i}$$

On the other hand, for goal as a target, the desirability ramps are created like a maximum on the way up, and a minimum on the way down.

Finally, for a goal within a range, the desirability will be defined by the following equation:

$$d_i = 0, \quad \text{for} \quad Y_i = \text{Low}_i$$

$$d_i = 1, \quad \text{for} \quad \text{Low}_i < Y_i < \text{High}_i$$

A value of D different to zero implies that all responses are in a desirable range simultaneously and consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so as the response values are near target values [38,41]. Table 5 shows the criteria for the optimization of each individual response in our study. As it could be seen the first goal was to minimize the retention time of MPA. As MPA is the last substance in the chromatographic run it determines the total analysis run time. The second goal was for retention factor of MPAG to be in the desired range (0.8–1.18) in order to insure good separation of MPAG from human plasma and urine peaks and not to prolonged total analysis run time. Considering proposed goals the individual desirability functions (d_i) for each goal and then the over all desirability function were obtained.

Before discussing the over all desirability function D, it is worthwhile analyzing the individual desirability functions (d_i) in order to obtain the description of the system. The single-response surfaces are not very useful for finding the optimal conditions but can provide us with information considering desirable chromatographic behavior of our substances. The response surfaces corresponding to d_{MPA} and d_{MPAG} are presented in Fig. 3. Considering MPA (Fig. 3a), the d is approaching to 1 when percentage of acetonitrile is approaching to 35% and if at the same time pH of the water phase is approaching to 4.40. The interaction of percentage of acetonitrile and pH of the water phase is obviously present. The influence of pH of the water phase is much greater on the retention time of MPA if working with lower percentage of acetonitrile.

Considering MPAG (Fig. 3b), the interaction between examined variables also exists and the influence of acetonitrile on the retention factor of MPAG is much grater when working with lower pH of the water phase. The values of d approaching to 1 are with 25% of acetonitrile and pH of 2.4.

As we were expected the experimental conditions when d is approaching to 1 are completely different for MPA and MPAG due to their differences in polarity. For final choice of optimal conditions, which would satisfy all proposed goals, the over all Derringer's desirability function is obtained and is graphically presented at Fig. 4. The influence of acetonitrile and pH of the water phase could be easily noticed. When working with lower percentage of acetonitrile the influence of pH is mush greater. Also, if pH is lower the influence of acetonitrile on D function has greater impact. The satisfactory chromatographic conditions which would fulfill both goals could now be selected and are thoroughly presented in Section 2.3.

3.3. Method validation

Bioanalytical method validation was performed according to FDA [30] and ICH [31] guidelines. The validation of the proposed method has already been reported [42] and therefore the validation parameters are here briefly summarized and presented in Tables.

To insure the selectivity of the method the blank urine and plasma obtained from ten volunteers (different age and sex) were chromatographically screened for interfering substances. No coelution was observed at the retention times of MPA, MPAG and internal standard comparing with freshly prepared spiked samples

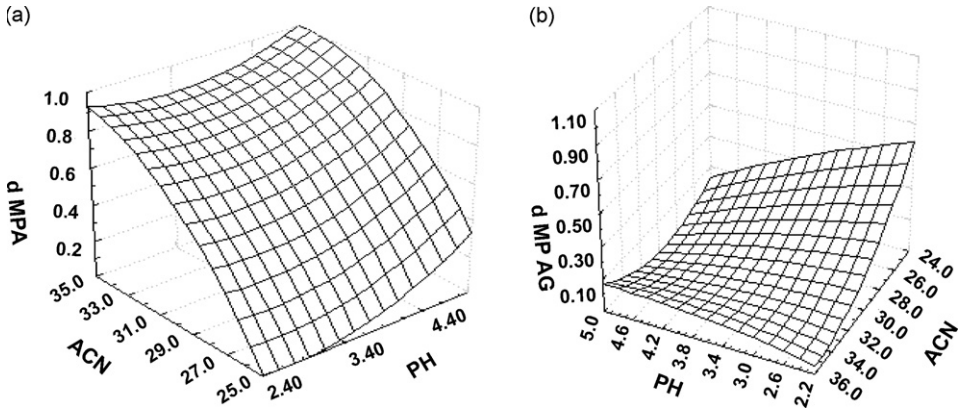

Fig. 3. Response surface plots of the individual desirability functions (d_i) for MPA (a) and MPAG (b).

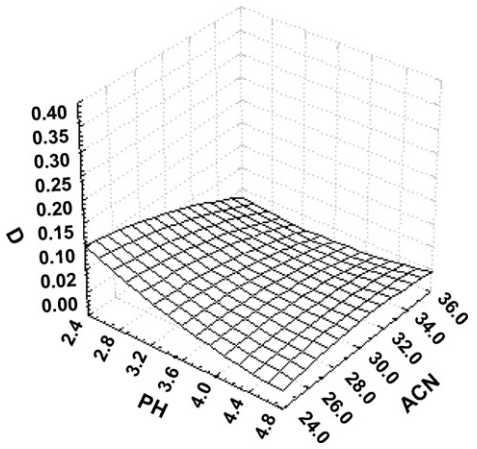

Fig. 4. Response surface plots of over all desirability function *D* for proposed goals.

at LLOQ level. The corresponding chromatograms of blank plasma and urine samples are presented in Fig. 5A and Fig. 6A.

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 6. 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.

LOD values were obtained experimentally. For MPA it showed to be 0.1 μg/mL and for MPAG 0.2 μg/mL in both plasma and urine matrixes. 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 met all required criteria and was accepted as limit of quantification and firs point on calibration curve. Concentrations 1 μg/mL for MPA and 1 μg/mL for MPAG, both in plasma and urine showed to be LLOQ.

The results for both precision and accuracy are presented in Table 7 for intra-day and in Table 8 for inter-day precision and accuracy. All presented data indicates that method parameters meet all FDA and ICH required criteria.

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 patient's plasma and urine samples are presented in Fig. 5C and Fig. 6C, respectively. Along with mycophenolate mofetil, patients were co-administered 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. In order to ensure the selectivity of the method, as we worked with

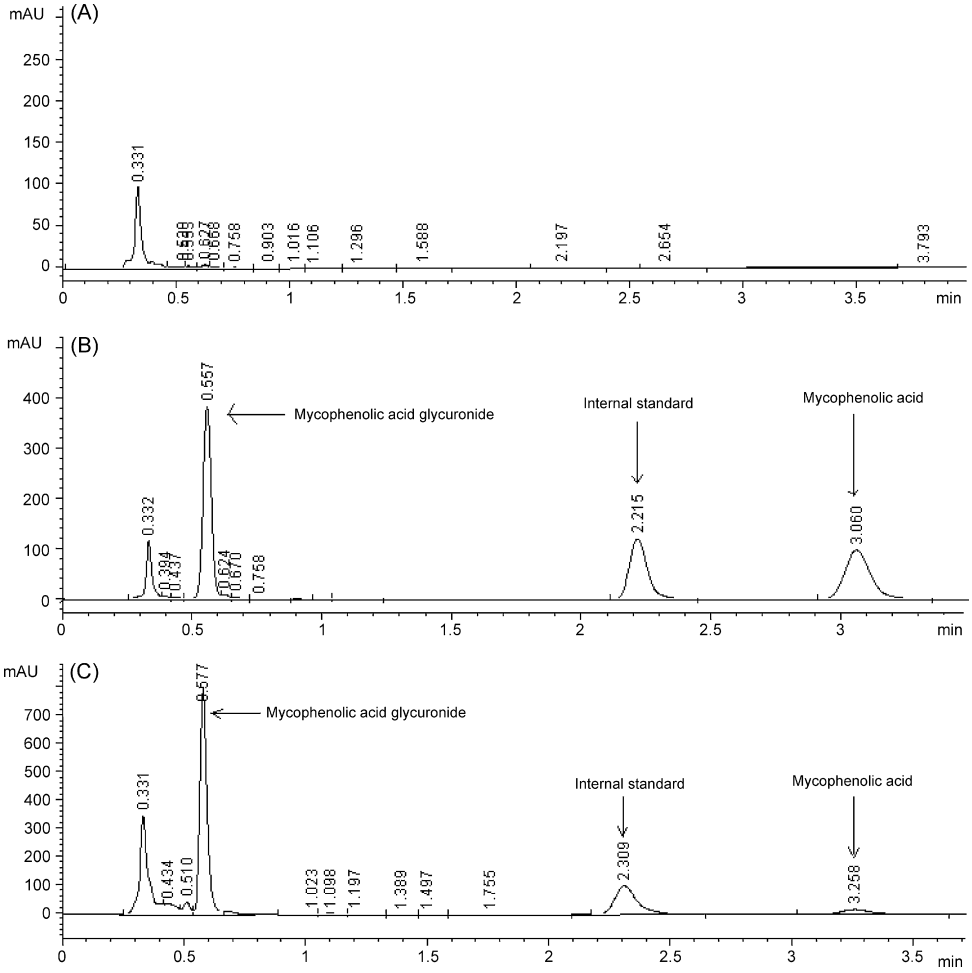

Fig. 5. The representative chromatograms of blank plasma (A), plasma samples spiked with 10 μg/mL of MPA, 20 μg/mL of MPAG and 50 μg/mL of internal standard (B), and plasma sample (3.15 μg/mL of MPA and 62.5 μg/mL of MPAG) of stable kidney transplant patient at 0.5 h after receiving an oral dose of 500 mg of MMF (C).

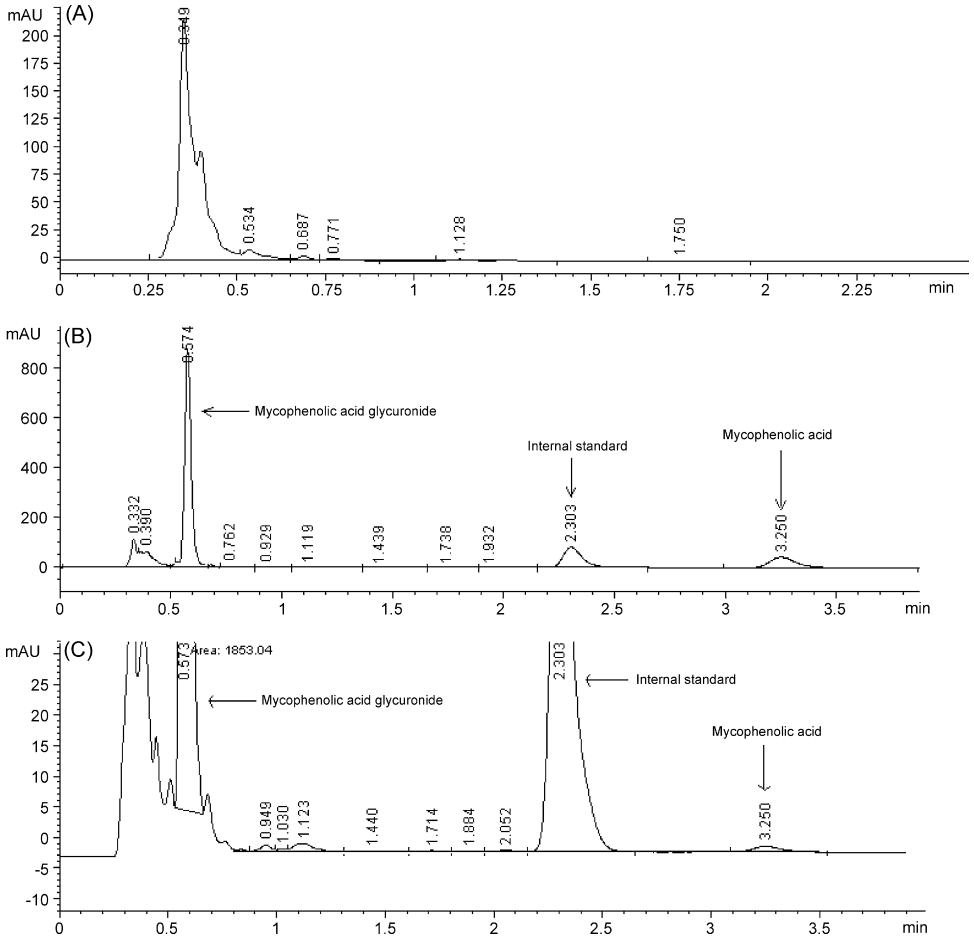

Fig. 6. The representative chromatograms of blank urine (A), urine samples spiked with 5 μg/mL of MPA, 50 μg/mL of MPAG and 50 μg/mL of internal standard (B), and urine sample (1.8 μg/mL of MPA and 54.85 μg/mL of MPAG) of stable kidney transplant patient at 4.0 h after receiving an oral dose of 500 mg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

Table 6
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.

Table 7
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 (RSD, %)	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 ((g/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 (RSD, %)	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.

Table 8
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 (RSD, %)	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 (RSD, %)	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.

no-selective UV detector, we investigated plasma and urine samples of the volunteers who were administered above-mentioned drugs except mycophenolate mofetil and no interferences were observed.

4. Conclusion

With assistance of experimental design and Derringer's desirability function new chromatographic method has been developed. Being sensitive, precise, accurate and rapid this method is suitable for rapid screening of MPA and MPAG concentrations in urine and plasma of pediatric patients after kidney transplantation. As pediatric population is well known as population with high variability, especially children with organ transplantation and possible organ failure, the method for rapid screening of MPA and MPAG concentrations is more than welcome in this area. The detailed optimization enabled us to investigate and determine complete chromatographic behavior of the investigated substances. Because of this, the presented paper could be valuable for further investigation and establishment of other chromatographic methods.

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