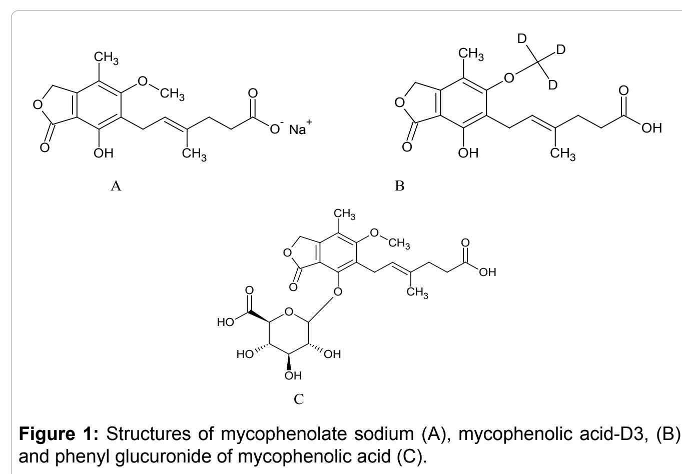

Figure 1: Structures of mycophenolate sodium (A), mycophenolic acid-D3, (B) and phenyl glucuronide of mycophenolic acid (C).

Working solutions were prepared by appropriate dilution of stock solutions in acetonitrile. Calibration standards and quality control samples were prepared by adding 50 µL of the working solution to 950 µL of blank plasma. There are eight calibration concentrations: 0.5, 1.0, 4.0, 8.0, 12.0, 18.0, 24.0, 30.0 µg/mL, and six quality control samples: 0.5, 1.5 µg/mL (LQC, low quality control), 5.0, 15.0 µg/mL (MQC, medium quality control), 22.5, 30.0 µg/mL (HQC, high quality control).

The test product was Myfortic 360 mg coated tablet from Novartis Pharma Stein AG, Switzerland (batch number: S0347, expiry date: 12.2014)

Analysis of plasma samples

The plasma samples (50 µL) were deproteinized with internal standard solution (1.5 µg/mL) in acetonitrile (450 µL) and 5 µL portions of supernatant obtained after centrifugation at a speed of 2500 rpm. were directly injected into the chromatographic system.

The chromatographic separation was achieved by using Phenomenex Kinetex C18 (30 mm × 4.6 mm, 2.6 µm) analytical column and Phenomenex Security guard C18 (4 mm × 3 mm) pre-column with gradient elution of the mobile phase composed of acetonitrile and water (Table 1) at a flow rate 0.4 mL/min and a room temperature.

Detection was performed (Table 2) in negative ion mode, using the Heated-ESI ion source. An MRM transition of 319→191+205 m/z was selected for the analyte, and 322→191+205 m/z for the internal standard.

Results

Method validation procedures

Validation was conducted in accordance with requirements of FDA [15] and EMEA guidelines [16] on the following parameters: selectivity, linearity, lower Limit of Quantification, accuracy and precision, matrix effect, recovery, carryover effect, dilution integrity and stability.

The chromatograms obtained with blank plasma (6 samples from independent sources, including haemolyzed and hyperlipidaemic plasma) did not have any interference at the retention time of the analyte and the internal standard (Figure 2). Therefore, the developed method is selective.

The linearity of analytical procedure was evaluated in the concentration range from 0.5-30.0 µg/mL by measuring area ratio response ("analyte/internal standard"). The correlation coefficient was ranged from 0.9985 to 0.9996. The lower limit of quantification (LLOQ) was 0.5 µg/mL.

Accuracy and precision were determined by analysing of six replicates of QC samples. The results are represented in Table 3 and Figure 3. Diluting plasma sample to half did not affect the accuracy and precision.

The MDA recovery rate was 84.18% and 90.33% at low and high concentration levels, respectively. Matrix effect was assessed by comparing the mean area ratio of plasma sample with mean area ratio of acetonitrile solutions of MDA and MDA-D3. The Normalized Matrix Factor (NMF) values were 0.892 and 0.877 at low and high concentration levels, respectively; the Coefficients of Variation (CV) for NMF were 11.94% and 1.92%, respectively.

The stability study was carried out at concentrations of 1.50 µg/mL and 22.50 µg/mL with six replicates of each level. Short-term, long-term, freeze and thaw stability was demonstrated. The results are represented in Table 4.

There was no significant carry-over after three high concentration injections: Chromatograms obtained with blank plasma did not have peaks at the retention time of the analyte and the internal standard.

Back-conversion of MPAG was evaluated after addition 50 µL of MPAG acetonitrile solution to 950 µL blank plasma. The concentration of MPAG at plasma samples was 100 µg/mL, which corresponds to a maximum expected concentration of this metabolite in the samples of volunteers. The comparing of concentration of MPA was carried out after preparation of MPAG samples and after 24 h of storage of MPAG samples. There was no significant back-conversion MPAG: concentration of MPA was 2.58% from LLOQ.

The results of all validation tests were acceptable.

The pharmacokinetic study

The investigation was conducted in accordance with requirements of the National Standard of the Russian Federation GOST R 52379-2005 "Good Clinical Practice requirements" [17], guidances of FDA [18] and EMEA [19] as well as in accordance with ethical principles of the Declaration of Helsinki [20].

Time (min)	Acetonitrile (% v/v)	Water (% v/v)
0–1.0	40	60
1.0–1.5	65	35
1.5–2.0	90	10
2.0–2.5	90	10
2.5–3.0	65	35
3.0–3.5	40	60
3.5–4.5	40	60

Table 1: Parameters of gradient elution.

Parameter	Value
Spray voltage	3250 V
Capillary temperature	222°C
Sheath gas	30 arb. unit
Sweep gas	2 arb. unit
Aux gas	20 arb. unit
Vaporizing temperature	324°C
Collision gas pressure	1.5 mTorr

Table 2: Parameters of mass spectrometry detection.

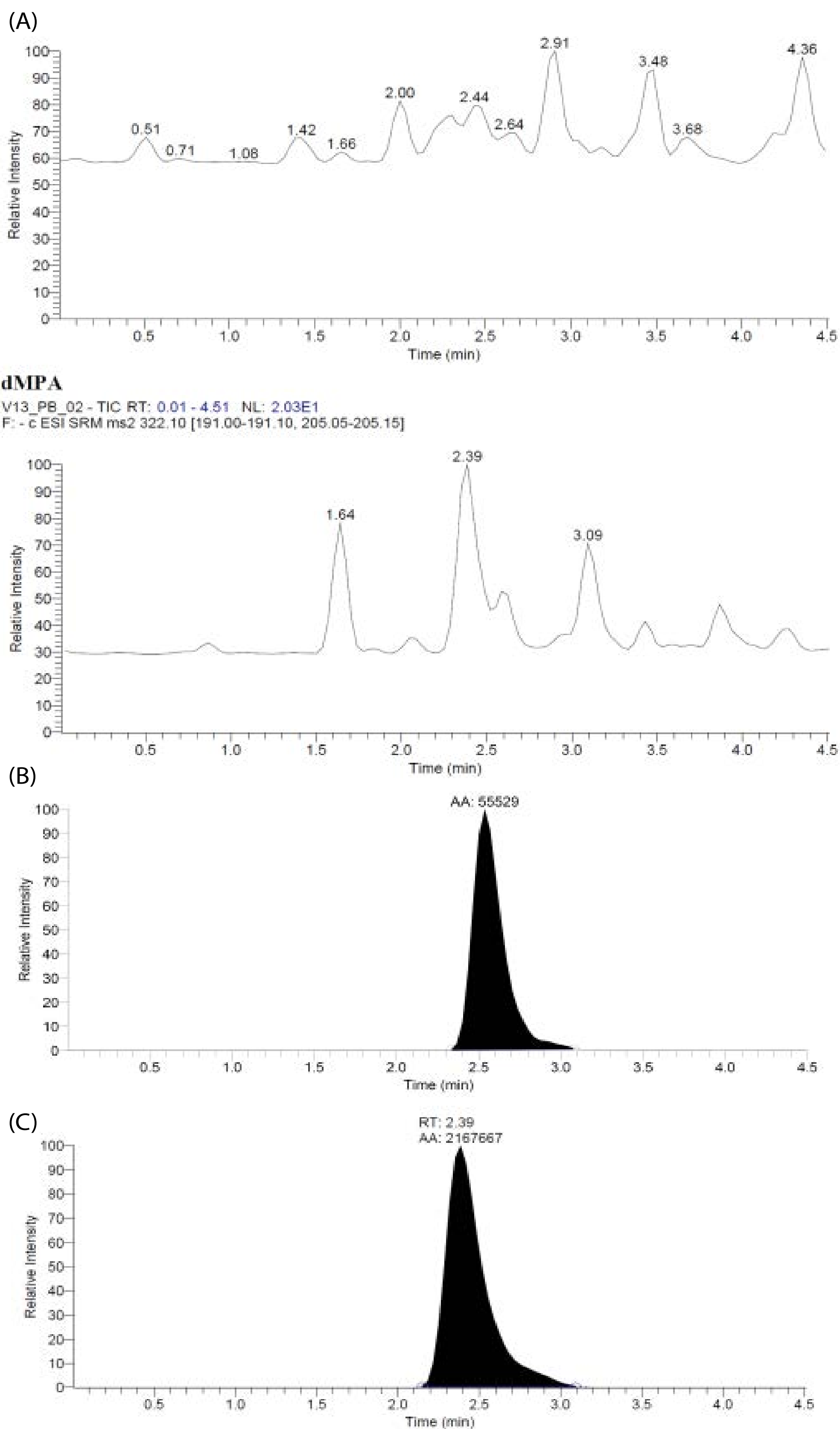

Figure 2: (A) The chromatograms of blank plasma. (B) Plasma with analyte. (C) Internal standard.

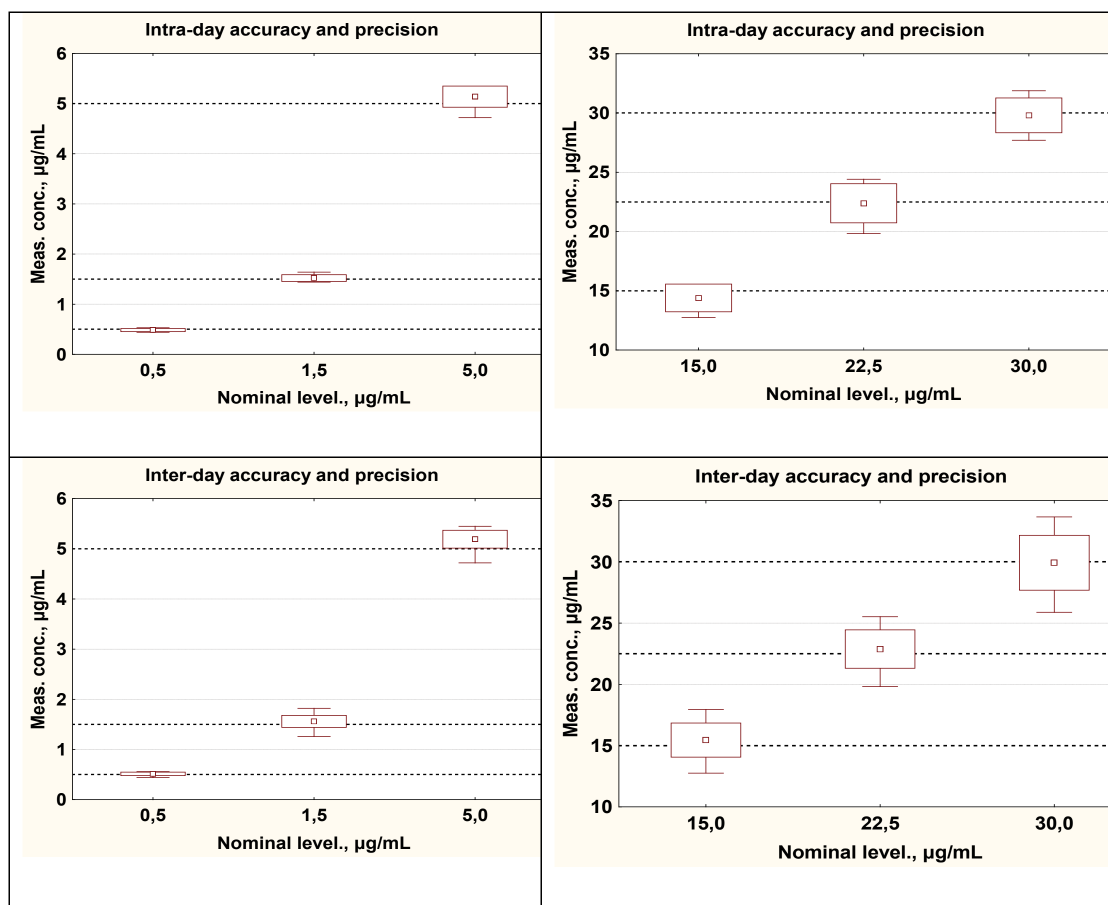

Figure 3: Intra- and inter-day accuracy and precision.

Concentration (µg/mL)	0.50	1.50	5.00	15.00	22.50	30.00
Intra-day accuracy and precision						
Mean (n=6)	0.54	1.67	5.33	16.93	24.29	31.91
SD	0.02	0.08	0.14	0.74	0.97	1.44
Precision (CV) %	4.42	4.62	2.54	4.36	4.01	4.52
Accuracy, %	108.62	111.38	106.67	112.85	107.96	106.37
Inter-day accuracy and precision						
Mean (n=18)	0.51	1.56	5.19	15.45	22.88	29.93
SD	0.03	0.12	0.18	1.39	1.57	2.24
Precision (CV)%	6.75	7.63	3.42	9.01	6.87	7.48
Accuracy, %	102.78	104.04	103.83	103.03	101.70	99.76

Table 3: Accuracy and precision for MDA QC samples.

	Short-term stability (24 h)	Long-term stability		Freeze and thaw stability
		37 days	119 days	
1.50 µg/mL				
Mean	1.49	1.51	1.55	1.58
Precision, %	4.04	5.72	4.23	5.47
Accuracy, %	99.18	100.38	103.24	105.10
22.50 µg/ml				
Mean	22.38	21.33	22.69	23.30
Precision, %	4.51	2.44	2.82	3.72
Accuracy, %	99.46	94.79	100.86	103.55

Table 4: Stability study of MDA at plasma.

The study was conducted on 48 healthy participants aged from 18 to 45 years that met the inclusion criteria: verified diagnosis "healthy", body mass index in the range of 18.5 kg/m^2 to 24.9 kg/m^2, body weight more than 45 kg; ability to follow the requirements of the study protocol, lack of allergy, normalities revealed by clinical instrumental and laboratory investigations at screening, lack of hypersensitivity to mycophenolate sodium, mycophenolate mofetil or to any other substance included in the formulation of the drug, lack of cardiovascular, bronchopulmonary, neuroendocrine, immune, gastrointestinal, liver, kidney, or blood diseases; lack of acute infectious disease within the 4 weeks preceding the investigation; lack of intake of any medicinal products within 2 weeks of the start of the study, lack of excessive alcohol consumption.

All participants were tested for use of drugs and alcohol. Women additionally performed a pregnancy test. Blood samples for subsequent quantitative determination of mycophenolic acid were collected into a pre-labelled vacuum centrifuge tubes containing EDTA as an anticoagulant prior to administrating the drug, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 18 h, 24 h, 48 h, 72 h after administration of a tablet of "Myfortic" at a dose of 360 mg [4]. After the blood sampling procedure, tubes were centrifuged at 2500 rpm for 10 min. The resulting plasma was immediately frozen at -20°C until it was assayed. There were taken and analysed 768 blood samples.

Pharmacokinetic parameters such as maximum measured plasma concentration (C_{max}), area under the pharmacokinetic "concentration-time" curve from zero to the last blood sampling procedure (AUC_{0-t}), relative absorption rate (C_{max}/AUC_{0-t}), time-to-peak concentration (T_{max}) were calculated. Statistical analyses performed with the Rv application packages 3.2.1, Module Bear (Lee, Hsinya and Lee, Yung-jin (2014), bear: Data Analysis Tool for Average Bioequivalence and Bioavailability, Rpackage version 2.6.4) and StatSoft Statistica v.12. The results are represented in Table 5 and Figure 4.

Conclusion

The new accurate, selective, fast method was developed for determining concentration of mycophenolic acid in blood plasma by

Parameters	C_{max}, µg/ml	T_{max}, h	AUC_{0-t}, µg ·h/ml	C_{max}/AUC_{0-t}, h^{-1}
M ± SD	12.29 ± 5.53	2.9 ± 2.4	22.90 ± 11.11	0.5591 ± 0.1812
Min	1.15	1.0	6.90	0.1667
Max	23.34	18.0	57.32	0.9939

Table 5: Pharmacokinetic parameters of coated tablet "Myfortic" 360 mg.

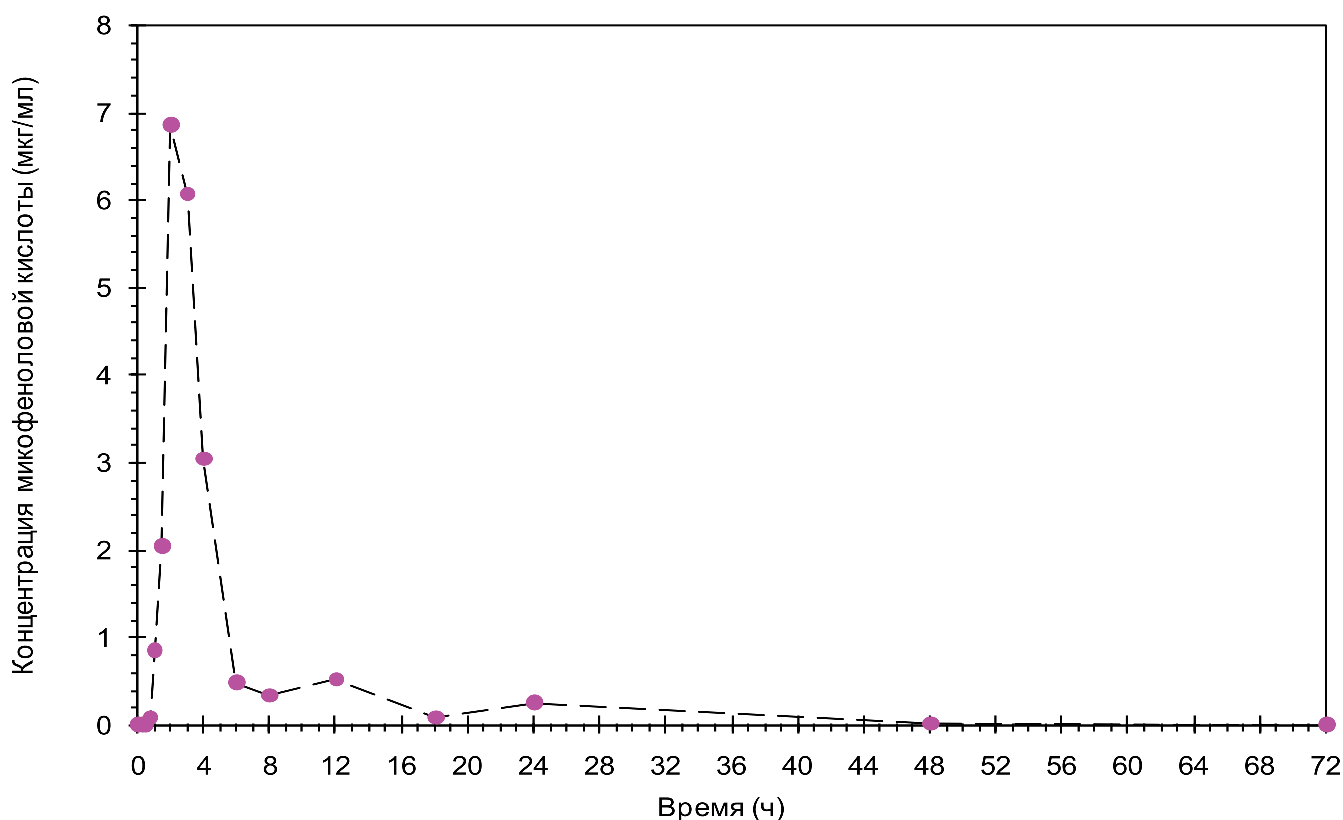

Figure 4: Averaged pharmacokinetic profiles of plasma mycophenolic acid concentrations after a single dose of "Myfortic".