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Journal of Chromatography B, 698 (1997) 293–300

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of mycophenolic acid and its glucuronide conjugate in human plasma by a single-run ion-paring method

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Received 23 January 1997; received in revised form 17 April 1997; accepted 1 May 1997

Abstract

A quick and robust HPLC method for simultaneous determination of plasma concentrations of mycophenolic acid (MPA) and its glucuronide conjugate (MPAG) is described. Using tetrabutylammonium dihydrogen phosphate as ion-paring reagent in the mobile phase, concentrations for both MPA and MPAG can be determined in a single HPLC run on a reversed-phase C_{18} column at 254 nm. The method is reproducible and accurate, with lower limits of quantification of 0.100 μg/ml for MPA and 0.800 μg/ml for MPAG. The quantification ranges of the method are 0.100–40.0 μg/ml for MPA and 0.800–400 μg/ml for MPAG using a 0.5-ml aliquot in the analysis. © 1997 Elsevier Science B.V.

Keywords: Mycophenolate mofetil; Mycophenolic acid; Mycophenolic acid glucuronide

1. Introduction

Mycophenolate mofetil (MMF, 1 in Fig. 1), the morpholinoethyl ester of mycophenolic acid (MPA, 2 in Fig. 1) is an immunosuppressant administered for the prevention of renal allograft rejection following organ transplantation [1,2]. In vivo, mycophenolate mofetil is rapidly absorbed and hydrolyzed to MPA [3,4], which is further metabolized to its glucuronide conjugate (MPAG, 3 in Fig. 1) [5]. MPA, the active immunosuppressive agent, is a potent and specific inhibitor of de novo purine synthesis and blocks the proliferation of both T and B lymphocytes [6,7]. MPAG is pharmacologically inactive but may be hydrolyzed in vivo to form free MPA.

To determine the pharmacokinetics of MPA and MPAG in the presence of potentially coadministered drugs, or in specific populations or disease states, quantification of both MPA and MPAG in plasma is needed. Several HPLC methods have been reported for determination of MMF, MPA and/or MPAG in human plasma [4,8–10]; however, none of these methods can quantify both MPA and MPAG in a single HPLC run. To accommodate both MPA and MPAG, which differ greatly in polarity, the method by Tsina et al. [8] requires a separate HPLC run for quantification of each analyte in plasma. Such a procedure becomes time consuming and labor intensive for analyzing a large number of samples. In order to increase sample throughput, a quick and robust analytical method was desired, especially for routine assay of plasma samples from large and

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0378-4347/97/$17.00
PII S0378-4347(97)00276-4

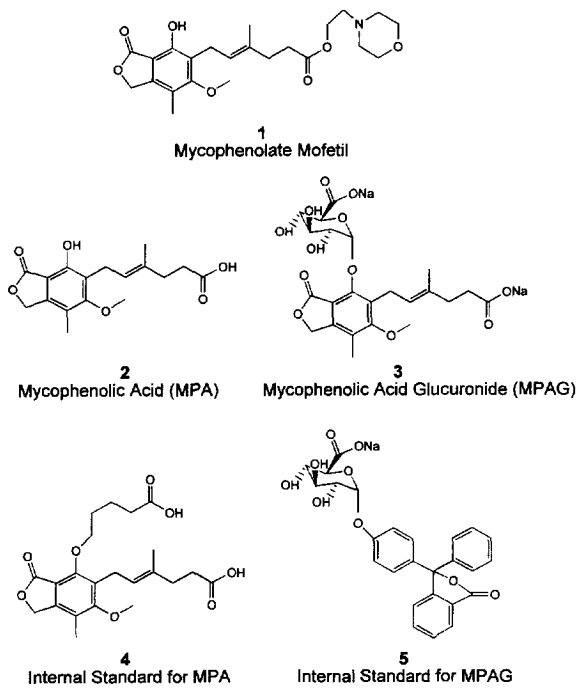

Fig. 1. Structures of mycophenolate mofetil, mycophenolic acid, mycophenolic acid glucuronide, and their internal standards.

long-term clinical studies. A critical goal was also that any revised method needed to be equivalent to the previous method and to replace it seamlessly. We report here the successful development of an HPLC method that allows simultaneous determination of concentrations of both MPA and MPAG in a single run. The improvement not only increases sample throughput but also reduces the cost of the assay by requiring less instrumentation, data processing and solvent waste control.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Burdick and Jackson Laboratory (Muskegon, MI, USA). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Reagent grade phosphoric acid, 85%, analytical-grade sodium acetate, potassium phosphate, glacial acetic acid, and 0.10 *M* hydrochloric acid were purchased from Mallinckrodt (St. Louis, MO, USA). Tetrabutylammonium dihydrogen phosphate was purchased from Aldrich (Milwaukee, WI, USA). Solid-phase extraction (SPE) columns, containing 200 mg of C_{18} sorbent, were purchased from Varian Sample Preparation Products (Harbor City, CA, USA). Mycophenolic acid, (*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate (MPA, 2 in Fig. 1), mycophenolic acid glucuronide disodium salt (MPAG, 3 in Fig. 1), and internal standard for MPA, (*E*)-6-[1,3-dihydro-4-(4-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl]-4-methyl-4-hexenoic acid (RS-60461-000, 4 in Fig. 1) were obtained from Syntex Research (Palo Alto, CA, USA). The internal standard for MPAG, phenolphthalein mono-β-glucuronic acid, sodium salt (5 in Fig. 1) was purchased from Sigma (St. Louis, MO, USA). Pooled human plasma (blank plasma) from normal volunteers was obtained from the Clinical Studies Unit (Syntex). 0.05% aqueous phosphoric acid and 0.1 *M* sodium acetate buffer (pH 4.0), were prepared in house.

2.2. Preparation of standard spiking solutions of MPA and MPAG

Separate stock solutions of MPA and MPAG were prepared by accurately weighing a portion of the compound and dissolving it in an appropriate amount of methanol to yield a final concentration of 1.00 mg/ml for MPA and 5.00 mg/ml for MPAG. The stock solutions were further diluted with 90% methanol in water to prepare spiking solutions at concentrations of 0.500, 1.00, 2.00, 3.00, 5.00, 10.0, 20.0, 50.0, 100 and 200 μg/ml for MPA and 4.00, 8.00, 15.00, 25.00, 50.0, 100, 200, 500, 1000 and 2000 μg/ml for MPAG. Stock internal standard solutions were prepared by dissolving the internal standards in methanol and were further diluted in 90% methanol in water to prepare a single spiking solution containing both internal standards for MPA and MPAG at concentrations of 1.00 μg/ml and 10.0 μg/ml, respectively. For preparation of the calibration standards used for construction of the calibration curve and for validation of the method, 0.100 ml of an MPA spiking solution and 0.100 ml of an MPAG spiking solution were added to a given 0.5-ml

aliquot of blank human plasma, so that the concentration ranges equivalent to 0.1–40 μg per ml of plasma for MPA and 0.8–400 μg per ml of plasma for MPAG were obtained.

2.3. Preparation of quality control samples

Quality control (QC) samples, prepared in bulk by spiking MPA and MPAG at four different concentrations into pooled blank plasma, were apportioned into aliquots and stored at −20°C. QCs were prepared at the following four concentrations: QC1 (MPA: 0.216 μg/ml; MPAG: 8.02 μg/ml), QC2 (MPA: 2.14 μg/ml; MPAG: 78.1 μg/ml), QC3 (MPA: 19.9 μg/ml; MPAG: 153 μg/ml), QC4 (MPA: 36.2 μg/ml; MPAG: 359 μg/ml). QC samples were stored frozen with study samples. To monitor the performance of the method, two QC samples at each of the four concentrations were analyzed with the study samples each time that the assay was carried out.

2.4. Sample extraction

2.4.1. Extraction of calibration standards

For each calibration standard, 1.5 ml of water was added to a 0.5-ml aliquot of blank plasma in a disposable glass tube, followed by addition of 0.100 ml of an MPA spiking solution, 0.100 ml of an MPAG spiking solution and 0.100 ml of the solution of internal standards. Then 0.75 ml of 0.1 *M* HCl solution was added. The mixture was vortexed briefly and applied to a C_{18} solid-phase extraction column which had been preconditioned with 2 ml of methanol followed by 2 ml of water. The column was allowed to drip dry by gravity. The test tube that contained the treated plasma was washed with 1 ml of 0.05% aqueous phosphoric acid, and the wash was applied to the same solid-phase extraction column. The column was then washed with 1 ml of 30% methanol in 0.05% phosphoric acid and allowed to drip dry. The eluate was discarded. The analytes and internal standards were eluted from the column with 1 ml of 80% methanol in 0.1 *M* sodium acetate buffer (pH 4.0). An aliquot of this extract was subjected to HPLC analysis (see below).

2.4.2. Extraction of clinical samples

Samples of the heparinized plasma obtained from study subjects were stored at −80°C prior to analysis. Samples were thawed for approximately 15 min in a 25°C water bath, vortexed for 30 s and centrifuged for 2 min at approximately 110–200 *g*. An aliquot of 0.5 ml was then used for analysis. The samples were extracted using the procedure described above for the calibration standards, except that there was no addition of MPA and MPAG spiking solutions. The concentrations of MPA and MPAG in the samples were calculated by reference to calibration curves generated from calibration standards analyzed along with the clinical samples.

2.5. Chromatographic conditions

A 45-μl aliquot of the final extract of calibration standard or study sample was subjected to HPLC on a reversed-phase C_{18}, BDS hypersil 4.6×150 mm, 5-μm column (Keystone Scientific, Bellefonte, PA, USA). The mobile phase consisted of premixed 29% acetonitrile and 71% of 25 m*M* potassium phosphate, 25 m*M* tetrabutylammonium dihydrogen phosphate, pH 6.6 (v/v). The flow-rate was 0.6 ml/min. A C_{18} guard column (Keystone Scientific) and a 0.094× 0.25 inch, 0.5-μm stainless steel frit precolumn filter (Upchurch Scientific, Oak Harbor, WA, USA) were connected to each analytical column (ambient temperature). A Rainin pump (Rainin Instrument, Wobum, MA, USA) was used for chromatography, and a Kratos Spectroflow 783 variable-wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ, USA) set at 254 nm was used for the detection and quantification of the analytes.

2.6. Data handling and calculations

Linear least-square regression was performed on the peak-height ratio versus concentration data generated by the calibration standards to construct a linear standard curve. Since our laboratories were not equipped with a chromatography data system capable of performing weighted linear regression analysis to construct the standard curve, calibration curves from 0.100–4.00 μg/ml for MPA and 0.800–40.0 μg/ml for MPAG were constructed using unweighted linear regression. The calibration standards of higher con-

centrations were used to verify extrapolations of the curve up to 40.0 μg/ml for MPA and 400 μg/ml for MPAG. A verification standard must be within 10% of its nominal value in order to quantify sample concentration in the extrapolation range in that run. This verification of the upper range of the calibration curve in every run avoided the dominating effect of the upper points on the low end of the unweighted standard curve and minimized the need for sample dilution in order to achieve concentrations within a narrow curve range. We continued this extrapolation procedure, which we had used for our previous method [8], because major goals for a new method were equivalence with the previous one and completely seamless replacement on our existing laboratory instrumentation. We suggest, however, that those who want to validate either this or the previous method for their own use should consider using weighted linear regression as an alternative to the extrapolation technique presented here.

3. Results and discussion

3.1. Sample purity

Prior to HPLC analysis, the analytes were extracted from plasma by solid-phase extraction according to the procedures described above in Section 2.4. A two-step wash procedure with 0.05% aqueous phosphoric acid followed by 30% methanol in 0.05% phosphoric acid was included. The wash steps added beyond those reported in the previous method [8] for sample preparation removed most water-soluble proteins and other potential interferences present in plasma, and therefore, provided much cleaner samples, smoother chromatography baselines and longer HPLC column life.

3.2. Chromatography of MPA and MPAG

Since MPA and MPAG differ greatly in polarity, the previous method [8] needed separate HPLC runs to quantify each analyte: a strong mobile phase (39% acetonitrile in 0.05% phosphoric acid) for the relatively non-polar MPA and a weak mobile phase (21% acetonitrile in 0.05% phosphoric acid) for the relatively polar MPAG. Such a procedure is quite time consuming and labor intensive for analyzing a large number of samples. The improvement of the method was achieved by two critical modifications of the mobile phase that were designed to reduce the difference in polarity of MPA and MPAG. First, the pH of the mobile phase was increased above the pK_a value of MPA (pK_a of the carboxylic acid group of MPA is 4.5) to improve the ionization of MPA, thereby increasing the polarity of MPA. As shown in Fig. 2A, at a mobile phase pH of 8.0, all four compounds eluted from the column in about 25 min. However, MPAG eluted too close to the solvent-

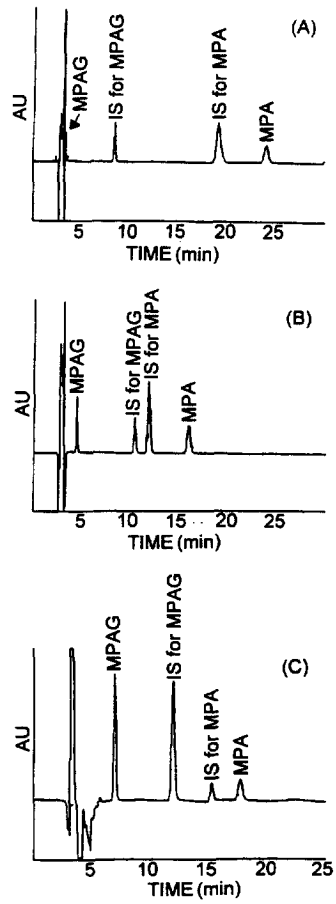

Fig. 2. Chromatograms for the separation of MPA, MPAG and their internal standards with different mobile phases. (A) 19% acetonitrile-81% 25 m*M* potassium phosphate (pH 8.0); (B) 25% acetonitrile–75% 25 m*M* potassium phosphate, 2.5 m*M* tetrabutylammonium dihydrogen phosphate (pH 7.3); (C) 29% acetonitrile–71% 25 m*M* potassium phosphate, 25 m*M* tetrabutylammonium dihydrogen phosphate (pH 6.6).

front, and the run time was still relatively long. The second step was to use tetrabutylammonium dihydrogen phosphate as an ion-pairing reagent to retain MPAG. Use of a 2.5 m*M* concentration of the ion-paring reagent showed a significant improvement in retaining MPAG and shortened the run time as well, as shown in Fig. 2B. Although the sample preparation procedure removed most of the water-soluble proteins, there was still a big solvent front peak due to the injection solvent and to interferences that remained in the sample. Therefore, it was desirable for the first peak of interest (MPAG) to elute as far away from the solvent front as possible without significantly increasing the run time. This was accomplished by increasing the concentration of tetrabutylammonium dihydrogen phosphate to 25 m*M* in the mobile phase (Fig. 2C). Increasing the concentration of tetrabutylammonium dihydrogen phosphate in the mobile phase also eliminated potential problems associated with using a low concentration of ion-paring reagent, such as irreproducible retention times and variation of response with the injected sample mass. The entire sample run time was 20 min and both MPA and MPAG concentrations were obtained in a single run. There were no endogenous interferences from blank plasma, and typical chromatograms are shown in Fig. 3.

3.3. Precision and accuracy of the method

The lower limit of quantification (LLOQ) of the method was 0.100 μg/ml for MPA and 0.800 μg/ml for MPAG with a signal-to-noise ratio of 80 and 500 for MPA and MPAG, respectively, using 0.5 ml of plasma in the analysis. Typical calibration curves for MPA and MPAG were generated with linear correlation coefficients of 0.9997 or greater (data not shown). The linear range can be extrapolated to 40.0 μg/ml for MPA and 400 μg/ml for MPAG. The precision was assessed by intra-assay and inter-assay coefficients of variation (% C.V.) of the method. The accuracy of the method was evaluated by the recovery, defined here as the ratio of the concentration of MPA or MPAG found to that added, expressed as a percentage. Data for the intra- and inter-assay percentage C.V. values and mean recoveries for

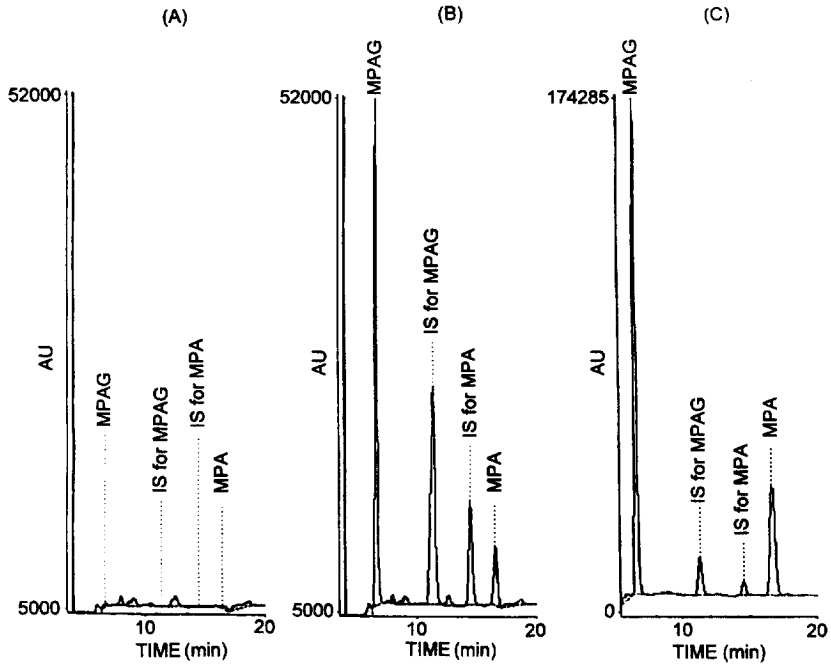

Fig. 3. Typical chromatograms generated with the HPLC conditions described in Section 2.5 for (A) blank human plasma; (B) blank plasma spiked with MPA (1 μg/ml), MPAG (10 μg/ml) and their internal standards; (C) a study sample.

Table 1
Intra- and inter-assay precision and accuracy of the method (for MPA)

Concentration of MPA spiked (μg/ml)		n		Mean concentration found (μg/ml)		C.V.(%)		Mean recovery (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Calibration standards									
0.1		4	1	0.112	–	4.54	–	111	–
0.1		4	4	0.202	0.198	1.40	1.52	101	98.8
0.4		4	4	0.404	0.396	2.76	1.51	101	98.9
0.6		4	4	0.600	0.606	0.840	0.83	99.8	101
1		4	4	0.972	0.988	6.65	2.29	97.3	98.8
2		4	4	2.04	2.00	0.940	1.53	102	100
4		4	2	4.08	1.03	0.630	1.75	102	101
10		4	4	10.3	10.0	0.820	2.09	103	100
20		4	2	20.2	19.7	2.74	3.37	101	98.7
40		4	2	40.1	41.1	1.87	0.34	103	103
QC samples									
QC1	0.216	4	4	0.215	0.211	3.44	3.57	99.5	97.7
QC2	2.14	4	4	2.16	2.13	2.62	1.18	101	99.3
QC3	19.9	4	4	20.4	19.9	0.800	4.77	103	99.7
QC4	36.2	4	2	36.0	36.5	1.20	1.94	99.4	101

calibration standards prepared and analyzed in addition to those used for construction of the standard curves are presented in Tables 1 and 2. The validity of the method was also assessed by analyses of quality control (QC) samples, which were prepared as described above by spiking a known amount of each analyte into pooled plasma from untreated subjects. The results obtained with QCs are also

Table 2
Intra- and inter-assay precision and accuracy of the method (for MPAG)

Concentration of MPAG spiked (μg/ml)		n		Mean concentration found (μg/ml)		C.V. (%)		Mean recovery (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Calibration standards									
0.8		4	1	0.72	-	1.64	-	90.3	-
1.6		4	2	1.76	1.64	2.46	10.3	110	102
3		4	4	3.04	3.01	0.76	4.68	101	100
5		4	4	5.13	5.00	2.07	1.99	103	100
10		4	4	10.3	10.0	1.76	3.06	103	100
20		4	4	20.4	20.3	1.79	1.03	102	102
40		4	4	39.8	39.9	1.48	0.24	99.5	99.7
100		4	4	91.2	93.8	2.35	2.22	91.5	93.8
200		4	2	185	194	3.92	6.44	92.4	97.0
400		4	3	383	404	7.32	4.95	95.8	101
QC samples									
QC1	8.02	4	4	7.79	7.59	3.44	3.91	99.5	94.6
QC2	78.1	4	4	70.6	70.6	2.62	0.75	101	90.4
QC3	153	4	4	140	149	0.800	3.83	103	97.1
QC4	359	4	2	339	361	1.20	8.34	99.4	100

presented in Tables 1 and 2. For both calibration standards and for QCs, all percentage C.V. values were less than 8%. All mean recoveries were between 91.5% and 103%, except for the calibration standard with MPA and MPAG concentrations at the respective LLOQs, which had a mean recovery of 111% for MPA and 90.3% for MPAG.

Although the aglycon portion of the MPAG internal standard differs structurally from that of MPAG itself (Fig. 1), the suitability of the internal standard was substantiated for this method and previous method [8] by consistent and accurate MPAG quantification of QC samples in hundreds of assay runs, as well as by consistent and accurate MPAG quantification for both calibration samples and QC samples during method validation.

3.4. Stability

As reported by Tsina et al. [8], MPA and MPAG were stable in whole blood, plasma and cell fractions at 1–4°C for at least 8 h. Both analytes in QC samples were stable for at least 11 months when stored in a −20°C freezer. After thawing samples, MPA and MPAG were stable in plasma subjected to three freeze/thaw cycles. After extraction from plasma, MPA and MPAG were stable in the extract and could be stored for at least 6 days at room temperature (20–23°C) under normal laboratory conditions or for at least 2 weeks when refrigerated (1–4°C) prior to analysis by HPLC.

3.5. Comparison with previous method

The method reported here was used to analyze 48 samples that had been analyzed using the method with two separate HPLC runs [8]. Ninety-six percent of the results determined by the single-run method were within 10% of the values reported by the previous method for both MPA and MPAG. The results were compared by performing a linear regression analysis of the two sets of data (Fig. 4). The regression lines have slopes near 1.0, intercepts near zero, and correlation coefficients greater than 0.98, which indicate excellent agreement between the results obtained by the two methods.

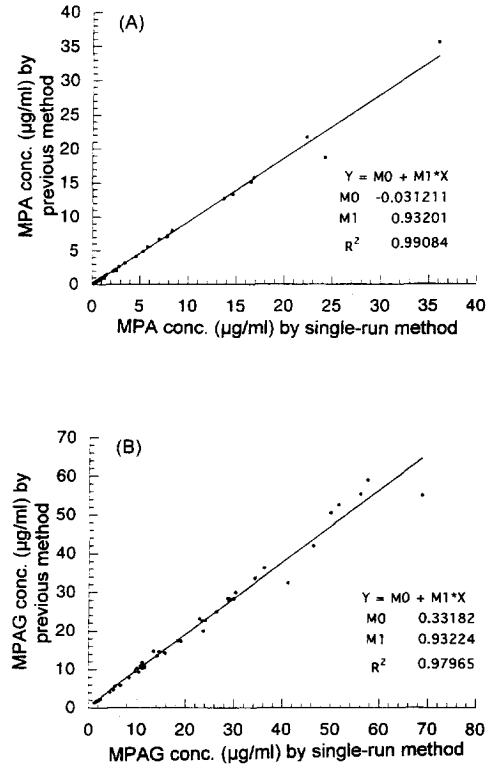

Fig. 4. Correlation of results obtained with the method described here (single-run method) and previously reported method for (A) MPA and (B) MPAG.

4. Conclusions

The method described here for quantification of MPA and MPAG in human plasma is specific, precise and accurate. The total run time is 20 min, which eliminates the need for two separate instruments in parallel or one in sequence for both analytes, so that total instrument time per sample is decreased by more than 10 min. Therefore, the new method not only increases sample throughput, but also reduces the cost of the assay by requiring less instrumentation, maintenance and waste control. Furthermore, the improved wash step during sample extraction decreases baseline noise and prolongs column life. The method can be used for samples obtained during clinical studies from subjects receiving mycophenolate mofetil, since the results correlated well with those obtained by the previously reported method [8].

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