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Short communication

Simple and sensitive high-performance liquid chromatographic method for the determination of mycophenolic acid in plasma

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Abstract

A rapid, selective, sensitive, and reproducible reversed-phase HPLC procedure for the quantitative determination of mycophenolic acid (MPA) — an active plasma metabolite of the immunosuppressant mycophenolate mofetil (MMF) in plasma is described. The procedure involves one-step extraction of MPA and the internal standard, standard [*RS*-60461-000: (*E*)-6-[1,3-dihydro-4-(4-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzo-furanyl-4-methyl-4-hexenoic acid] with dichloromethane–dichloroethane (1:1, v/v) at acidic pH. Chromatographic separation consisted of the mobile phase [acetonitrile–0.05% phosphate buffer, pH 3.4 (45:55, v/v)] running through the column (Techopak-10 C_{18}) at flow-rate of 0.8 ml/min. Detection was at UV wavelength of 254 nm. The mean recoveries of MPA and the internal standard at concentrations of 0.1 and 20 μg/ml were 89–98%, and 90–96%, respectively. The within-day coefficients of variation for MPA were 0.3–7.8% and the day-to-day coefficients of variation were 1.1–2.0%. The minimum detectable concentrations for both MPA and the internal standard in plasma were 0.005 μg/ml. The method was found to be suitable for use in clinical pharmacokinetic study.

Keywords: Mycophenolic acid

1. Introduction

Mycophenolate mofetil (MMF), an ester prodrug of the immunosuppressant — mycophenolic acid (MPA) has been approved for maintenance immunosuppressive therapy of allogeneic graft rejection following solid organ transplantation. MPA is a potent and specific inhibitor of de novo purine synthesis and blocks proliferation of both T and B lymphocytes [1–3]. Due to the rapid and extensive metabolism of MMF to the active plasma metabolite, pharmacokinetic investigation following the administration of the prodrug — MMF has been based principally on the kinetics of MPA. Since MMF is at the moment, at a relatively early stage of the drug development process, the full pharmacokinetic characterization in recipients of kidney transplantation, in conjunction with pharmacodynamics (clinical efficacy) are essential for optimization of drug therapy. To support this pharmacokinetic investigation, establishment of an appropriate analytical method (sensitive, selective, reproducible and simple) for quantification of MPA in biological fluids is essen-

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tial. A high-performance liquid chromatographic (HPLC) method with solid-phase extraction has recently been described for MPA [4]. In the present report, we describe an alternative method in which the extraction procedure is comparatively simpler, faster and cheaper.

2. Experimental

2.1. Chemicals

Standard powder of MPA [*RS*-5797-000: (*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate] and the internal standard (I.S.) [*RS*-60461-000: (*E*)-6-[1,3-dihydro-4-(4-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzo-furanyl-4-methyl-4-hexenoic acid] were obtained from Syntex (Technology Center, MA, USA) (Fig. 1). Stock solutions were prepared by dissolving MPA and the I.S. in absolute methanol. The stock solution was then further diluted with 90% methanol (v/v) to prepare spiking solutions of 0.5, 1, 2, 3, 5, 10, 25, 50 and 100 μg/ml for MPA. Spiking solution containing 10 μg/ml of the I.S. was prepared by further diluting the stock solution with 90% methanol. All standards were stored at 4°C in glass vials.

Absolute methanol, dichloromethane, dichloroethane and acetonitrile were of HPLC grade (Fisons, Loughborough, UK). All other reagents and solvents were analytical grade quality, and were supplied by BDH.

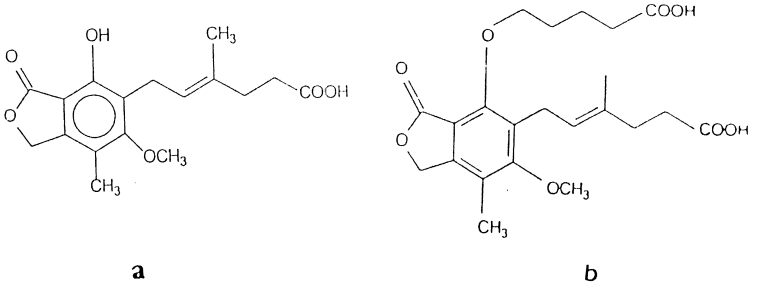
2.2. Chromatography

The method was developed on a liquid chromatographic system consisting of a Model 600 solvent delivery system (Waters, MA, USA), a Waters 740 data module (Waters) equipped with a Rheodyne 7125 injector with a 20-μl loop (Rheodyne, Berkeley, CA, USA), and a 486 variable-wavelength UV detector (Waters). The wavelength was set at 254 nm and sensitivity at 0.005 a.u.f.s.

The separation was carried out on a Techopak-10 C_{18} reversed-phase column (10 cm×4.6 mm I.D., 10 μm particle size; HPLC Technology, serial no. pp 45739). The mobile phase consisted of 0.05 *M* phosphate buffer (pH 3.2)–acetonitrile (45:55, v/v), delivered at a flow-rate of 0.8 ml/min. The chromatographic analysis was operated at ambient temperature.

2.3. Sample extraction procedure

In order to minimize eventual drug adsorption, extraction was carried out in 15-ml screwcap glass test tubes precoated with dimethyldichlorosilane in toluene (5%, v/v). To a 0.5-ml plasma sample, was added an I.S. (1 μg), followed by vortex mixing for 30 s. Hydrochloric acid (0.01 *M*, 1 ml) was added and the mixture was vortex-mixed for 30 s. The resultant mixture was extracted with 6 ml of dichloromethane–dichloroethane (1:1, v/v) by mechanical tumbling for 30 min. After centrifugation at 1500 *g* for 20 min (4°C), the clear organic layer was transferred to a clean tube using a pasteur pipette. Evaporation to dryness was by a stream of air at



a b

Fig. 1. Chemical structures of (a) MPA and (b) the internal standard.

37°C. The residue was dissolved in 100 μl of mobile phase, and 20 μl was injected onto the column.

2.4. Calibration curves

Solutions of MPA in 90% methanol, ranging from 0.1 to 100 μg/ml were injected into the HPLC system in order to assess detector linearity. Peak height was plotted against the quantity of MPA injected. MPA was linear ($r>0.999$) in the concentration range observed.

Calibration curves were prepared by triplicate analysis of 0.5-ml plasma samples spiked with 0.2 ml each of the MPA working solutions to obtain the concentration range of 0.1–20 (0.1, 0.2, 0.4, 0.6, 1, 2, 5, 10, 20) μg/ml, with a fixed concentration of I.S. (1 μg). Samples were analyzed as described above, and the peak height ratios of MPA to the I.S. were plotted against the corresponding drug concentrations. Linear least-squares regression was performed on the peak-height ratio vs. concentration data generated by the calibration standards, to construct a linear standard curve of MPA from peak height ratio$=m$ (concentration of MPA)$+b$. Peak-height ratios of the samples were determined and the concentrations calculated from the standard curves.

2.5. Method recovery, precision, accuracy, limit of quantification, stability and selectivity

The analytical recoveries of the extraction procedure for both MPA and the I.S. were estimated by comparing the peak heights obtained from an extracted sample with those measured with equivalent amounts of each compound in methanol. The concentrations used were 0.1, 1, and 20 μg/ml.

The minimum detectable concentration was defined as a peak height that produces three times of baseline noise at 0.0005 a.u.f.s.

The precision of the method based on within-day repeatability was determined by replicate analysis of five samples spiked with four different concentrations of MPA (0.1, 1, 10, 20 μg/ml). The reproducibility (day-to-day variation) of the method was established using the same concentration range as above, but only a single determination of each concentration was made on 5 different days. Coefficient of variation (C.V.) was calculated from the ratio of standard deviation (SD) to the mean.

Accuracy was determined by replicate analysis of four different levels (0.1, 1, 10, 20 μg/ml) and comparing the differences between spiked value and that actually found.

The stability of MPA and the I.S. was determined by storing spiked plasma samples (concentrations: 0.1, 1, 10, 20 μg/ml) at room temperature (25°C) for 48 h, and at −80°C for 6 months. Concentrations were measured periodically (2 weeks, 1, 2, 4 and 6 months).

The selectivity of the method was verified by checking for interference by commonly coadministered drugs (cyclosporine, prednisone, methylprednisolone) after subjecting them to the extraction procedure.

2.6. Application of the method to biological samples

In order to validate the assay method, the method was applied for the pharmacokinetic investigation of MPA in a Thai male recipient of kidney transplantation (aged 24 years, weighing 52 kg) following the administration of a single oral dose of 1 g MMF (Hoffmann-La Roche, Basle, Switzerland; 250 mg per capsule). The study was approved by the Ethics Committee of Pramongkutklao Medical College. Venous blood samples (5 ml each) were collected into sodium heparinized plastic tubes at 0 (pre-dose), 20, 40, 75 min, and 2, 4, 6, 8 and 12 h after drug administration. Plasma samples were obtained through centrifugation (1500 *g*, 10 min), and stored at −80°C until analysis.

Pharmacokinetic analysis was carried out by model-independent method [5]. The maximum plasma concentration (C_{max}) and the time to maximum concentration (t_{max}) were observed values. The terminal phase elimination rate constant (λ_z) was determined by least squares regression analysis of the post absorption and distribution plasma concentration–time data, and the terminal phase elimination half-life ($t_{1/2z}$) from the ratio $0.693/\lambda_z$. The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal rule.

3. Results and discussion

Several methods for quantification of MMF, MPA and the MPA glucuronide inactive metabolite (MPGA) have been reported. These include bioassay, thin-layer chromatography (TLC) [6], gas chromatography (GC) [7,8], and HPLC [4,9–11]. HPLC methods involve the methods applied for fermentation broth and biological fluids. The early method reported by Lee et al. [9] for determination of MPA in biological fluids requires a 5-ml sample volume to determine the plasma MPA concentration, and therefore is not suitable for application to pharmacokinetic studies. A selective and sensitive isocratic HPLC assay using a relatively small volume of samples was later developed by Sugioka et al. [10] for the determination of MMF, MPA and MPGA in rat plasma, bile and tissue homogenate, and human plasma. The detection limit for all compounds is 0.17 μg/ml. Recently, a manual and an automated HPLC method for separate determination of plasma MMF, MPA and MPGA have been described [4,11]. The methods involve the quantification of MMF in plasma by solid-phase extraction using a 0.5-ml plasma sample. Both are reproducible and accurate, and are equivalent in all aspects, including quantification limits (MMF 0.4 μg/ml, MPA 0.1 μg/ml; MPAG 4 μg/ml), precision and accuracy.

In the present study, we describe a simple, sensitive and specific HPLC method using liquid–liquid extraction. Chromatograms of extracts of drug-free plasma, spiked plasma and plasma obtaining from a Thai male recipient of kidney transplantation at 2 h post-dosed with a single oral dose of MMF (1 g) are shown in Fig. 2. The I.S. and MPA were resolved with the retention times of 8.4 and 9.7 min, respectively. Endogenous peaks from extracted drug-free plasma did not interfere with drug analysis. The minimum detectable concentration, defined as a peak three times baseline noise at 0.005 a.u.f.s. in a 0.5-ml plasma sample was 0.005 μg/ml for both MPA and the I.S. Analytical recoveries were 89–98%, and 90–95% for MPA and I.S., respectively, at concentration range of 0.1–20 μg/ml.

Calibration curves for MPA were linear over the range of 0.1–20 μg/ml, with correlation coefficient of 0.9999 or better. Little variation in MPA assays was observed; coefficients of variation in all cases

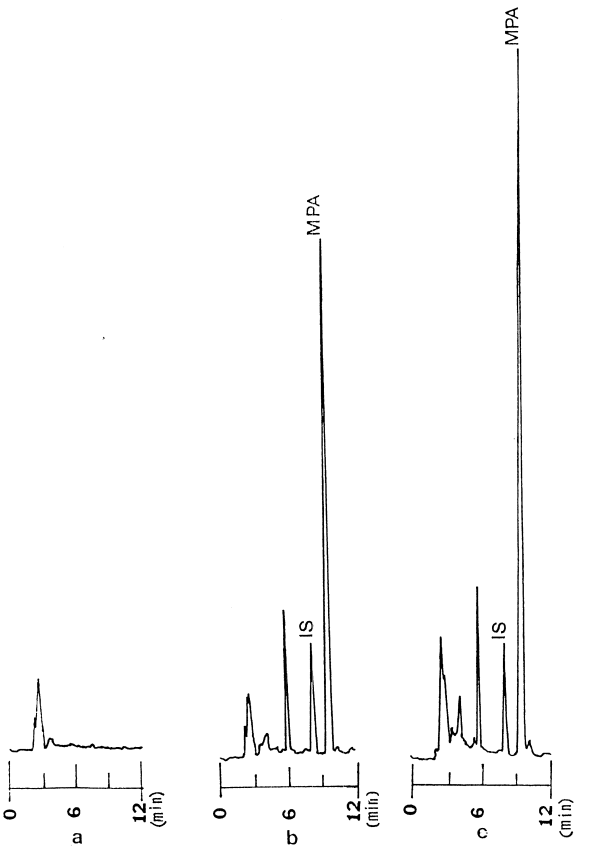


Fig. 2. Chromatograms of extracts of (a) drug-free plasma (b) spiked plasma (2 μg MPA, 1 μg I.S.) and (c) plasma obtained from a Thai male recipient of kidney transplantation at 2 h after a single oral dose of 1 g MMF (retention times of I.S. and MPA were 8.4 and 9.7 min, respectively).

were below 10%. The intra-assay (within-day) and inter-assay (day-to-day) variation for MPA at four different concentrations are given in Table 1.

Plasma samples containing MPA (0.1, 1, 10, 20 μg/ml) were found to be stable when stored at room temperature (25°C) for at least 28 h, and in a −20°C freezer for at least 6 months without significant decomposition of the drug.

To validate the clinical applicability of the method, the pharmacokinetics of MPA was investigated in a Thai male recipient of kidney transplantation following an oral administration of 1 g MMF. Plasma concentration–time profile is shown in Fig. 3. The profile is generally in agreement with those reported in previous studies [1–3]. MPA was rapidly

Table 1
Precision and accuracy for MPA assay in plasma ($n=5$)

Concentration added (μg/ml)	Concentration measured (mean±SD) (μg/ml)	C.V. (%)	Difference between measured and added concentration (%)
Within-day variation			
0.1	0.11±0.002	1.8	−0.1
1	1.02±0.03	2.9	+0.01
10	10.8±0.09	0.8	−0.05
20	19.95±0.06	0.3	−0.08
Day-to-day variation			
0.1	0.13±0.009	6.9	+0.1
1	1.1±0.05	4.5	+0.09
10	10.4±0.5	4.8	+0.18
20	19.8±0.25	1.3	+0.12

absorbed from the gastrointestinal tract: C_{max} (9.56 μg/ml) was attained at 1.25 h (t_{max}) of dosing. Plasma concentrations were measurable up to 12 h. AUC and $t_{1/2z}$ were 33.9 μg·h/ml and 19.1 h, respectively. Further investigation for detailed pharmacokinetics of MPA in Thai recipients of kidney transplantation is required for dose optimization.

In conclusion, the analytical method for the determination of MPA presented in this paper meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study. The advantages of the method over previously reported ones are basically, its simplicity and high sensitivity. In addition, the extraction procedure is simpler, faster and less expensive compared to the solid-phase cartridge extraction previously described.

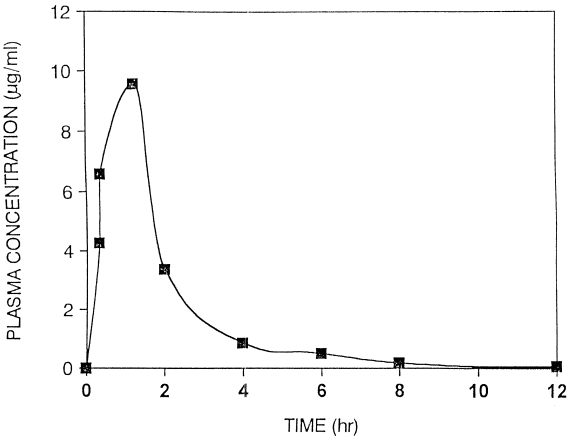


Fig. 3. Plasma concentration–time profile of MPA in a Thai male recipient of kidney transplantation following a single oral dose of 1 g MMF.

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