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Short communication

Simultaneous determination of mycophenolic acid and its phenolic glucuronide in human plasma using an isocratic high-performance liquid chromatography procedure

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Abstract

Simultaneous determination of mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG) in plasma was accomplished by isocratic HPLC with UV detection. After protein precipitation and phase separation with saturated sodium dihydrogenphosphate, chromatographic separation was achieved on a monolithic column "Chromolith Performance RP-18e", with acetonitrile/0.01 M phosphate buffer, pH 3, (25:75, v/v), as the mobile phase; flow rate 3.3 ml/min and measurement at 214 nm. Linearity was verified up to 40 mg/l for MPA and up to 400 mg/l for MPAG. Detection limits based on the analysis of 50 μl plasma were 0.05 and 0.5 mg/l for MPA and MPAG, respectively. Accuracy was 99.6–104% for MPA and 95.6–105% for MPAG and total imprecision (CV) was <7% for both compounds. Analytical recovery was >95% for MPA and MPAG. The method is simple, rapid, accurate and suitable for routine determination of MPA and MPAG in plasma.

Keywords: Mycophenolic acid; Mycophenolic acid glucuronide

1. Introduction

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), is an antiproliferative immunosuppressive agent, increasingly used after solid organ transplantation and also proposed for therapy of several autoimmune diseases [1–3]. Its immunosuppressive action resides in the noncompetitive, selective and reversible inhibition of inosine monophosphate dehydrogenase, thereby suppressing the de novo synthesis of guanosin nucleotides in T and B lymphocytes. This leads to an arrest of proliferation and function of these cells [4].

MPA is primarily metabolized by glucuronidation at the phenolic hydroxyl group to a mycophenolic acid glucuronide (MPAG), which is the major urinary excretion product of the drug [1,3]. Analysis of MPAG is of interest, as it achieves plasma levels many fold higher than MPA and is involved in enterohepatic recycling of MPA which results in secondary peaks in the concentration versus time profile and prolongs the apparent half-life of MPA in vivo. In addition to the pharmacologically inactive primary metabolite MPAG, evidence has been provided for the formation also of the acyl glucuronide and the phenolic glucoside metabolites [5]. Only the acyl glucuronide metabolite inhibits human inosine monophosphate dehydrogenase in vitro [5].

Several studies have demonstrated a relationship between MPA pharmacokinetics and clinical outcome, suggesting that monitoring plasma concentrations may be useful: to ensure that the immunosuppressive drug is not below threshold concentrations to prevent acute rejection, and to follow special populations where pharmacokinetics parameters have been shown to vary greatly (e.g., children, patients with altered absorption, dietary factors, drug–drug interactions) [3].

High-performance liquid chromatography (HPLC) is the method of choice for the quantitative determination of MPA and its main metabolite MPAG, and a number of methods

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have been developed [6–16]. Compared with the procedure described here, most of them require larger sample volumes [8,11–14,16] and more complex sample preparation procedures as liquid–liquid [6–7] or solid phase extractions [8–9], complicated post-column derivatization [10], mobile phase gradient elution [11,16], or dual analytical columns for MPA and MPAG [8]. Only few of the assays allow simultaneous determination of MPA and MPAG under isocratic conditions and UV detection [12–15].

Here we describe a quick and reliable reverse-phase HPLC method for the simultaneous determination of MPA and MPAG in plasma, developed on a novel monolithic stationary phase. The simple sample preparation, avoidance of the laborious and time-consuming extraction step, low sample volume required for the assay, good reproducibility and isocratic separation represent an advance of this method over the other already published methods for routine therapeutic drug monitoring.

2. Experimental

2.1. Materials

Sodium dihydrogen phosphate, phosphoric acid and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). MPA and MPAG were obtained from Hoffmann-La Roche (Grenzach-Wyhlen, Germany) and the internal standard—carboxy butoxy ether of MPA (MPAC) was kindly supplied to us by Dr. Maria Shipkova (Georg-August University, Göttingen, Germany). The purity of the standards used was over 98%.

2.2. Instrumentation

The HPLC system consisted of mostly Waters (Milford, MA, USA) components, including a model 510 pump, a 717 plus autosampler, a 996 photodiode array detector, and a computer interface system controller linked to a PC.

The Millenium software (Waters, Milford, MA, USA), version 2.10, was used for recording and calculating the data. Calculations were made in the internal standard mode using peak area ratios.

2.3. Preparation of standards and quality controls

Stock solutions of MPA and of the internal standard MPAC were prepared separately in acetonitrile each at a concentration of 1 g/l and stored at −20 °C. The MPAG stock solution (4 g/l) was prepared in acetonitrile/water (80/20, v/v) and also stored at −20 °C. Seven calibration standards ranging from 0.1 to 40 mg/l MPA (0.1, 0.5, 1, 5, 10, 20 and 40 mg/l) and from 1 to 400 mg/l for MPAG (1, 5, 10, 50, 100, 200 and 400 mg/l) were prepared diluting the stock solution in drug free plasma and stored in 50 μl aliquots at −20 °C. Quality controls were prepared in nominal concentrations of 0.5, 1, and 10 mg/l for MPA and 10, 40, and 200 mg/l for MPAG and also stored in 50 μl aliquots at −20 °C.

2.4. Patients' samples

EDTA blood samples were obtained from 60 kidney transplant patients, receiving MMF and co-medications such as cyclosporine A, tacrolimus and prednisolone for immune suppression. After centrifugation at 3000 × *g* for 5 min plasma was derived and either immediately analyzed or stored at −20 °C until measurement.

2.5. Sample preparation and HPLC analysis

For analysis, 50 μl of plasma (calibrator, control or patient derived), 50 μl of saturated sodium dihydrogenphosphate and 100 μl of acetonitrile containing the internal standard MPAC (5 mg/l) were pipetted into an Eppendorf microcentrifuge tube. The sample was vortex-mixed for 20 s, and then centrifuged for 3 min at 10,900 × *g*. An 80 μl aliquote of the supernatant were evaporated to dryness under a stream of nitrogen, reconstituted with 80 μl of mobile phase, and 10 μl of the latter solution were injected for analysis.

Chromatographic separation was performed on a monolithic column Chromolith Performance RP-18e (100 mm × 4.6 mm), (Merck, Darmstadt, Germany), maintained at 40 °C in an Eppendorf CH-80 column heater (Hamburg, Germany). The mobile phase consisted of acetonitrile/0.01 M phosphate buffer, pH 3 (25:75, v/v). The flow rate was 3.3 ml/min and the compounds were quantified at 214 nm. Each chromatographic run was completed in 8 min.

3. Results

3.1. Chromatography

Representative chromatograms of a plasma sample, obtained from a renal transplant patient not on MMF therapy (A) and a sample containing 1.8 mg/l MPA and 170 mg/l MPAG, obtained from renal transplant patient on MMF therapy immediately before a dose (B) are shown in Fig. 1. Retention times of MPAG, MPA and IS (MPAC) were 0.93, 5.56, and 6.63 min, respectively. As depicted, the substances eluted fully baseline-separated as symmetric peaks, which facilitated their subsequent quantification.

A small interfering peak in the area near the MPAG was observed as well as in patients not on MMF therapy as in patients on MMF. This peak was equivalent to approximately 0.2 mg/l, which is more than 100 times less than typical therapeutic levels of MPAG and therefore does not represent a significant interference in the analysis.

3.2. Linearity

Linearity of the assay was verified 1–40 mg/ml for MPA and 1–400 mg/l for MPAG (seven different concentrations,

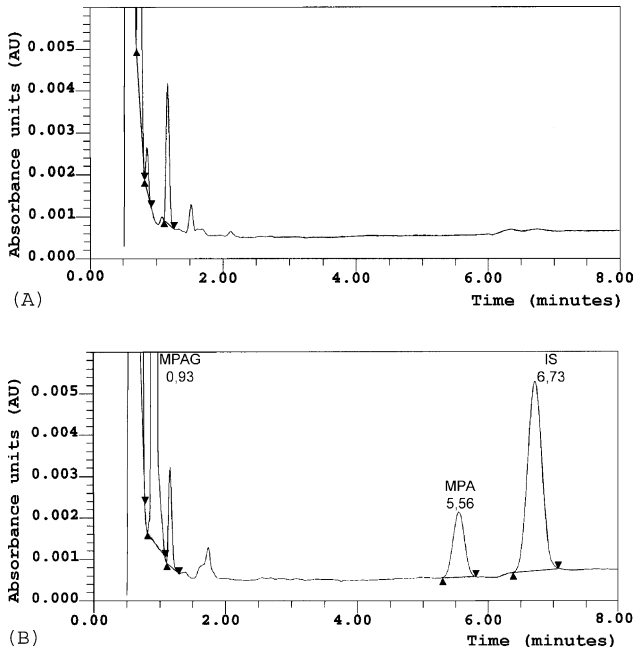

Fig. 1. Representative chromatograms of a plasma sample, obtained from a renal transplant patient not on MMF therapy (A), and a sample, containing 1.8 mg/l MPA and 170 mg/l MPAG, obtained from renal transplant patient on MMF therapy (2 g/24 h) immediately before a dose (B). Internal standard (MPAC, 5 mg/l) was added to the plasma sample shown in (B).

duplicate analysis) and standard curves were constructed using weighted $1/x^2$ linear regression. A typical calibration line gave a regression of $y = -0.0057 + 0.1556x$ for MPA ($r > 0.999$) and $y = 0.0059 + 0.0246x$ for MPAG ($r > 0.999$), where y is the relative peak area and x is the concentration (mg/l).

3.3. Limit of quantification and detection limit

The limit of quantification, defined as the lowest amount quantitatively determined in a sample of 50 μl, with a between-day precision of five replicates around the mean value less than 10% was 0.1 mg/l for MPA and 1 mg/l for MPAG. At these concentrations, both the imprecision and accuracy between run were less than 8.5%. These values are comparable to that previously reported by other authors [15,16], even though a smaller supernatant volume was injected in the study reported here.

The limit of detection, defined as the concentration that produces a signal-to-noise ratio of 3 for MPA was 0.05 and 0.5 mg/l for MPAG using 50 μl of plasma for analysis.

3.4. Precision, accuracy and extraction recovery

Within-run and between-run imprecision, accuracy, as well as the extraction recovery were studied with control plasma samples, spiked with three different concentrations of the analytes: low (MPA: 0.5 mg/l, MPAG: 10 mg/l), medium (MPA: 2.0 mg/l, MPAG: 40 mg/l) and high (MPA: 10 mg/l, MPAG: 200 mg/l). Within-run validation was conducted by preparing 12 separate samples of the three quality controls and analyzing them in 1 day. Between-run validation was performed by repeated analysis of these samples over 12 days. The imprecision and accuracy data of the assay are summarized in Table 1. The total coefficient of variation (CV) for both MPA and MPAG was less than 8% over the three concentration levels studied.

Accuracy of the method, expressed as a percentage of the mean assayed concentration over the weighed-in concentration, ranged from 99.6 to 104%, and 95.6 to 104.9% for MPA and MPAG, respectively. The extraction recovery, calculated by comparing peak areas obtained from the extracted plasma samples with MPA and MPAG added with peak areas obtained with solutions in mobile phase, containing the same amount of the compounds which were directly injected onto the column without extraction was over 95% for both compounds ($n = 5$).

3.5. Specificity

The specificity of the assay was evaluated, as multidrug use is quite common in patients undergoing organ transplantation. The potential chromatographic interferences from co-administered immunosuppressives (prednisolone, cyclosporin A, sirolimus, tacrolimus), antibiotics (amikacin, amoxicillin, cefazolin, chloramphenicol, gentamicin, tobramycin, vancomycin), NSAIDs (acetaminophen, salicylate), anticonvulsants (carbamazepine, phenobarbital,

Table 1
Within-run and between-run variability and accuracy of the HPLC assay for the quantification of MPA and MPAG in human plasma

Spiked concentration[a] (mg/l)	Mean ± S.D. (mg/l)		Coefficient of variation (%)		Accuracy (%)	
	Within-run ($n = 12$)	Between-run ($n = 12$)	Within-run ($n = 12$)	Between-run ($n = 12$)	Within-run ($n = 12$)	Between-run ($n = 12$)
MPA						
0.5	0.51 ± 0.01	0.52 ± 0.02	2.14	3.32	102.0	104.0
2.0	2.03 ± 0.07	2.04 ± 0.07	3.43	3.50	101.5	102.0
10.0	10.28 ± 0.17	9.96 ± 0.19	1.93	1.94	102.8	99.6
MPAG						
10.0	10.43 ± 0.21	10.49 ± 0.24	2.14	2.33	104.3	104.9
40.0	41.74 ± 1.03	41.86 ± 1.67	2.48	3.99	104.4	104.6
200.0	207.24 ± 4.31	208.88 ± 4.75	2.08	2.28	96.5	95.6

[a] Concentration of control samples for MPA and MPAG.

phenytoin) and other drugs as digoxin, cefazolin, cimetidine, clemastine, clonazepam, diazepam, lidocaine was examined by analysis of patient specimens received for routine therapeutic drug monitoring, TDM quality control sera (Riqas, Randox), and dissolved in 50% methanol drug standards to yield final concentration of each drug at 5 and 50 mg/l.

In addition, the existence of endogenous chromatographic interferences was evaluated by separate analysis of 50 patient specimens of transplant patients under immunosuppressive therapy without MMF, sent to the laboratory for routine clinical chemical tests.

Under the conditions of the assay described no extra peaks from endogenous compounds and various drugs, tested were observed at the retention times of MPA and MPAG.

3.6. Stability

Storage and analysis of the control plasma and patient samples over several months provided no evidence of apparent change of MPA or MPAG concentrations when stored at −20 °C. This is in agreement with MPA stability in plasma as reported earlier by Shipkova and co-workers [17] and Pawinski and Shaw [18].

3.7. Clinical application

Over a period of 1 year, the method has been successfully applied for the determination of MPA and MPAG levels in 60 renal transplant recipients on MMF therapy. Overall about 230 analyses were done. Predominantly samples were taken pre-dose, kept frozen and analyzed in series as part of the validation process. Unfortunately it was not possible to collect enough samples for constructing of concentration–time profile for MPA and MPAG in plasma. Therapeutic monitoring of MMF therapy is not a standard procedure in our country and after validation of this simple and reliable method for determination of MPA and MPAG in plasma, it will be already possible to develop a program for monitoring of patients on MMF after transplantation and we hope to be able to publish patient results in the nearest future.

4. Discussion

The purpose of the present study was to develop an HPLC-linked method for the simultaneous determination of MPA and its main metabolite MPAG in a single chromatographic run under isocratic conditions. The presented method requires only 50 μl sample volume, takes 30 min for preparation of a batch of 20 samples and 8 min for a single chromatographic development. In contrast to previously published HPLC methods, we succeeded to achieve comparable assay performance characteristics (Table 1), using a smaller sample volume, extremely simple sample preparation procedure and good chromatographic separation within a shorter run time. The Chromolith Performance column used is based on a new sol–gel process for the preparation of monolithic porous silica rods using highly pure metal free alkoxysilanes. The highly porous skeleton of this column allows operating at higher flow rates without loss of performance and limitations due to the column backpressure. The monolithic columns have demonstrated not only comparable repeatability and reproducibility to particle packed columns, but also a very easy handling on conventional HPLC systems and a very good stability [19–20].

In conclusion, we report a new simple, rapid and reliable HPLC method for the simultaneous determination of MPA and MPAG in human plasma, which is easily adaptable in many laboratories using commonly available HPLC equipment.

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