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Short communication

High-performance liquid chromatographic assay with a simple extraction procedure for sensitive quantification of mycophenolic acid in rat and human plasma

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Abstract

We describe a novel sensitive and simplified gradient HPLC assay for quantification of the immunosuppressant mycophenolic acid (MPA) in rat and human plasma. In contrast to previously reported MPA assays, our method used a single step extraction comprising addition of acetonitrile, which contained phenolphthalein glucoronic acid as internal standard, for protein precipitation. Linearity: 0.1–100 μg/ml (r^2>0.999), mean recoveries: MPA 98.0%, internal standard 105.2%, mean intra-day precision: 4.3%, mean day-to-day precision: 4.3%, mean day-to-day accuracy: −1.5%. Sensitivity was sufficient to allow for quantification of mycophenolic acid in as little as 50 μl plasma.

Keywords: Mycophenolic acid

1. Introduction

Mycophenolate mofetil (MMF), which is rapidly hydrolyzed after oral intake to the active compound mycophenolic acid (MPA), is used in combination with cyclosporine or tacrolimus as an immunosuppressant after organ transplantation [1]. Although several clinical studies demonstrated potential benefit of therapeutic drug monitoring for MMF, its impact on the clinical management of transplant patients and on the long-term outcome of transplantation is still under investigation [2]. In preclinical animal trials correlating pharmacokinetic and pharmacodynamic parameters, new pharmacodynamic monitoring strategies are studied. In these trials, specific HPLC assays are the method of choice for the quantification of MPA. However, most currently available HPLC assays for MPA require large sample volumes of 250 μl to 500 μl [3–5], which limits preclinical pharmacokinetic or pharmacodynamic studies to expensive and labor-intensive large animal models. The small blood volume in rodents demands analytical methods that can handle small sample volumes. In addition, most HPLC methods have only been validated for the quantification of MPA concentrations in human plasma [6,7]. Sugioka et al. [8] described an HPLC assay for MPA in rat plasma, which

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requires a plasma volume of only 50 μl. The extraction procedure, however, which includes several extraction steps, is laborious and time-consuming.

Pharmacokinetic studies for MPA or MMF in rats over 12 h or a longer period have not been published. Sweeney et al. published MPA concentrations 90 min after oral dosing of 45 mg/kg MPA as high as 10 μg/ml [9]. Sugioka and coworkers [8,10] whose HPLC assay had a lower limit of quantitation (LLOQ) of 0.1 μg/ml, reported plasma MPA levels below 1 μg/ml already 6 h after oral dosing of 16.6 mg/kg MMF. We estimated that for 48-h pharmacokinetic studies, concentrations as low as 0.1 μg/ml can be expected after 40 mg/kg MPA doses.

It was our objective to develop a simple and fast HPLC method with a LLOQ as low as 0.1 μg/ml to quantify MPA concentrations in small plasma volumes of 50 μl as required for pharmacokinetic studies in rodents.

2. Experimental

2.1. Materials and equipment

MPA and the internal standard phenolphthalein glucoronic acid were purchased from Sigma Chemicals (St. Louis, MO, USA). MPA-glucoronide (MPAG) and MMF were a gift from Roche (Basel, Switzerland). Acetonitrile (HPLC grade) was from Baxter (Deventer, The Netherlands), water (HPLC grade) from Fischer (Fair Lawn, NJ, USA), and plasma from Lewis rats from Accurate Chemical & Scientific Corp. (Westbury NY, USA). Human plasma was prepared by centrifuging heparinized blood drawn from healthy adult male volunteers. HPLC vials, inserts and teflon screw caps were purchased from Chromatography Research Supplies (Addison, IL, USA). The analytical column (200×4.6 mm) was filled with sphereclone, 5 μm ODS(2) material (Phenomenex, Torrance, CA, USA). Samples were analyzed on a binary HPLC-system equipped with an autosampler (Shimadzu, Kyoto, Japan).

2.2. Internal standard

The internal standard phenolphthalein glucoronic acid was dissolved in acetonitrile resulting in a final concentration of 25 μg/ml. The internal standard solution was stored in a freezer at −20°C in a light protected bottle.

2.3. Sample extraction

To 50 μl plasma, 100 μl of the internal standard solution was added for protein precipitation. Samples were vortexed for 20 s and centrifuged at 4800 *g* for 10 min. To 100 μl supernatant, 100 μl water was added. Samples were transferred to HPLC vials with 250-μl conical inserts and sealed with teflon screw caps.

2.4. HPLC analysis

Forty μl of the sample was injected into the HPLC system. Samples were eluted using the following acetonitrile–sulfuric acid (pH 4.0) gradient: 0 min: 20% acetonitrile, 10 min: 42% acetonitrile, 15 min: 42% acetonitrile, and 20 min: 20% acetonitrile. The flow-rate was 1.5 ml/min and the column temperature was maintained at 31°C. The column temperature was controlled to increase reproducibility. Since the column thermostat did not possess cooling capabilities and, therefore, could not stabilize temperatures too close to room temperature, a column temperature of 10°C above room temperature was chosen. The total run time was 24 min. UV absorption was measured at 218 nm. The MPA concentrations were calculated using a calibration curve and were corrected using the internal standard (CLASS-VP software, version 4.2, Shimadzu, Kyoto, Japan).

2.5. Calibration samples and quality control samples

One hundred mg MPA was dissolved in 10 ml methanol (stock solution). Further dilutions were prepared using methanol–water (20:80, v/v). To 5 ml bulk rat plasma samples, the appropriate amount of MPA dilutions was added (final concentrations see below). The volume of the MPA solution added did not exceed 100 μl. To allow for equal distribution of MPA, bulk rat plasma was incubated at room temperature for 2 h. Fifty μl aliquots were transferred into polypropylene tubes and kept frozen at −80°C until analysis. The final concentrations in the 50-μl

samples were 0.75, 2, 20 and 90 μg/ml for quality control samples and 0.1, 0.2, 1, 10, 25, and 100 μg/ml for calibration control samples.

2.6. Method validation

2.6.1. Acceptance criteria

The assay was considered acceptable when the following predefined criteria were met: Correlation coefficient (r^2) of the calibration curve >0.99, lower limit of quantitation: ≤0.1 μg/l, absolute recovery ≥60%, intra-day and day-to-day precision (% CV) at each concentration ≤15%, and accuracy (mean deviation from the nominal value) within ±15% for both intra- and day-to-day accuracy.

2.6.2. Calibration curve

Linearity of the assay was evaluated running the calibration samples (n=6 for each concentration).

2.6.3. LLOQ

The lowest concentration that met the following criteria was accepted as the LLOQ: 80% of the samples analyzed had to be within ±20% of the nominal value, and precision and accuracy had to be better than 20%.

2.6.4. Day-to-day and intra-day precision and accuracy

Intra-day precision and accuracy were evaluated from the results of eight sets of quality control samples processed and analyzed on the same day. Day-to-day variability (precision) and accuracy was assessed by analyzing six sets of quality control samples on each of 3 days. One set of quality control samples comprised four samples containing 0.75, 2, 20, or 90 μg/ml MPA.

2.6.5. Recovery

The UV signal ratios between the analyzed plasma sample and the same amount of MPA injected as stock solution were calculated. Determination of the recovery was based on the quality control samples (n=6 for each concentration).

2.6.6. Specificity and interferences

The lack of matrix interference was established by analysis of blank samples (n=15). A carry-over effect was ruled out by alternate analysis of blank samples and samples containing 90 μg/ml MPA (n=10). A possible interference with the pro-drug MMF and the main metabolite MPA-glucoronide was assessed by injecting 10 μg (10 μl of a 1 mg/ml stock solution in methanol) into the HPLC system (n=6).

2.6.7. Dilution integrity and partial volume verification

To establish dilution integrity, five plasma samples containing 100 μg/ml MPA were diluted five-fold and ten-fold using rat plasma. For partial volume verification, 25 μl of five 50-μl plasma samples containing 1 μg/ml MPA was extracted and analyzed.

2.6.8. Stability

Stability of MPA and the internal standard in the auto-sampler over 48 h was established. Two sets of quality control samples were extracted together and placed into the auto-sampler. One set was analyzed immediately, the other set was analyzed after staying in the auto-sampler at room temperature for 48 h (n=5).

2.6.9. Statistics

SPSS version 8.0 (SPSS Corp., Birmingham, AL, USA) was used for statistical analysis.

2.7. Animal study

Six adult male Lewis (RT1l) rats weighing 300–350 g (LEW/CrlBR, viral antibody free; Charles River Laboratories, Wilmington, MA, USA) were housed in microisolation cages. Food and water was provided ad libitum. The study was approved by the Stanford Institutional Animal Care and Use Committee and was carried out in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

MPA (20 mg/ml) was suspended in 0.2% sodium carboxymethyl cellulose solution (viscosity of 1300–2200 centipoises at 25°C in a 1% solution, City Chemical Corp., New York, NY). Blood samples were collected by retro-orbital bleeding under ether anesthesia before and 0.5, 1, 2, 6, 12, 24, 36 and 48 h after a single oral 40 mg/kg MPA dose. Blood

samples were anti-coagulated with 100 U heparin/ml, centrifuged and the plasma was stored at −80°C until analysis 4 weeks later.

3. Results

MPA eluted from the analytical column with a retention time of 15.7 min and the internal standard with a mean retention time of 10.6 min. Representative chromatograms after extraction of 50-μl samples of rat and human plasma are shown in Fig. 1.

3.1. Rat plasma

3.1.1. LLOQ and linearity

An MPA concentration of 0.1 μg/l was the lowest concentration that met the predefined criteria for LLOQ. Five out of six samples were within ±20% of the nominal value. Precision (CV) at the LLOQ ($n=6$) was 17.4%, accuracy 13.3%. The assay was linear from 0.1 to 100 μg/ml MPA with $y=30.08$ (± 0.33)$\cdot x-0.04$ (± 0.04) (mean±SD, $n=6$) and $r^2>0.999$. Precision at the upper limit of quantitation (100 μg/ml) was 1.9 %, and accuracy 1.6%.

3.1.2. Intra-day and day-to-day precision and accuracy

The intra-day precision/accuracy for plasma samples ($n=8$) containing 0.75 μg/ml MPA was 8.4%/–2.7%, for 2 μg/ml 4.3%/–1.8%, for 20 μg/ml 2.0%/–4.3% and for 90 μg/ml 2.5%/–3.0%. The day-to-day precision/accuracy was 8.5%/–1.6% for 0.75 μg/ml MPA samples, 4.6%/–0.1% for 2 μg/ml MPA samples, 1.7%/–3.0% for 20 μg/ml MPA samples and 2.5%/–1.4% for 90 μg/ml MPA samples. Analysis of variance (ANOVA) did not show any significant differences among the results obtained on different days.

3.1.3. Recovery

The mean (±SD, $n=6$) recovery from blood was 96.9% (±0.3%) for 0.75 μg/ml MPA samples, 95.9% (±2.5) for 2 μg/ml MPA samples, 99.5% (±2.0) for 20 μg/ml MPA samples and 99.5% (±1.7) for 90 μg/ml MPA samples. The overall mean recovery of MPA was 98.0%. The mean recovery of the internal standard from plasma was 105.2% ($n=18$).

3.1.4. Specificity and interferences

No peaks interfering with MPA or the internal standard were present in blank blood samples and no carry-over effect was observed (Fig. 1). The MPA pro-drug MMF (mean retention: time 17.8 min) and the major metabolite MPA-glucoronide (mean retention time: 10.2 min) did not interfere with either MPA or the internal standard.

3.1.5. Dilution integrity and partial volume verification

The mean %-deviation from the nominal value of the five-fold dilution was 2.4±4.1% and for the ten-fold dilution was 2.2±4.1% (mean±SD, $n=5$). The mean %-deviation from the nominal value of the 25-μl partial volume plasma sample was −2.9±4.0% (mean±SD deviation, $n=5$).

3.1.6. Stability

Extracted samples were stable in the auto-sampler (room temperature) for at least 48 h. The mean deviation from the immediately extracted and analyzed controls of the 0.75 μg/ml MPA samples was −3.2%, −1.8% for 2 μg/ml MPA samples, −3.9% for 20 μg/ml MPA samples and −5.3% for 90 μg/ml MPA samples ($n=5$).

3.2. Human plasma

In addition, in order to demonstrate suitability in humans, we validated our assay with human plasma. The mean intra-day precision was 2.5%, the mean day-to-day precision was 9.2%. Linearity (r^2) was better than 0.99. Six blank samples were tested (Fig. 1) and no interference with other peaks was observed. Again, a carry-over effect was ruled out by alternate analysis of blank samples and samples containing 90 μg/ml MPA ($n=6$).

4. Discussion

The method validation showed that our novel HPLC assay met all predefined acceptance criteria. Compared with a previously reported method [8], the

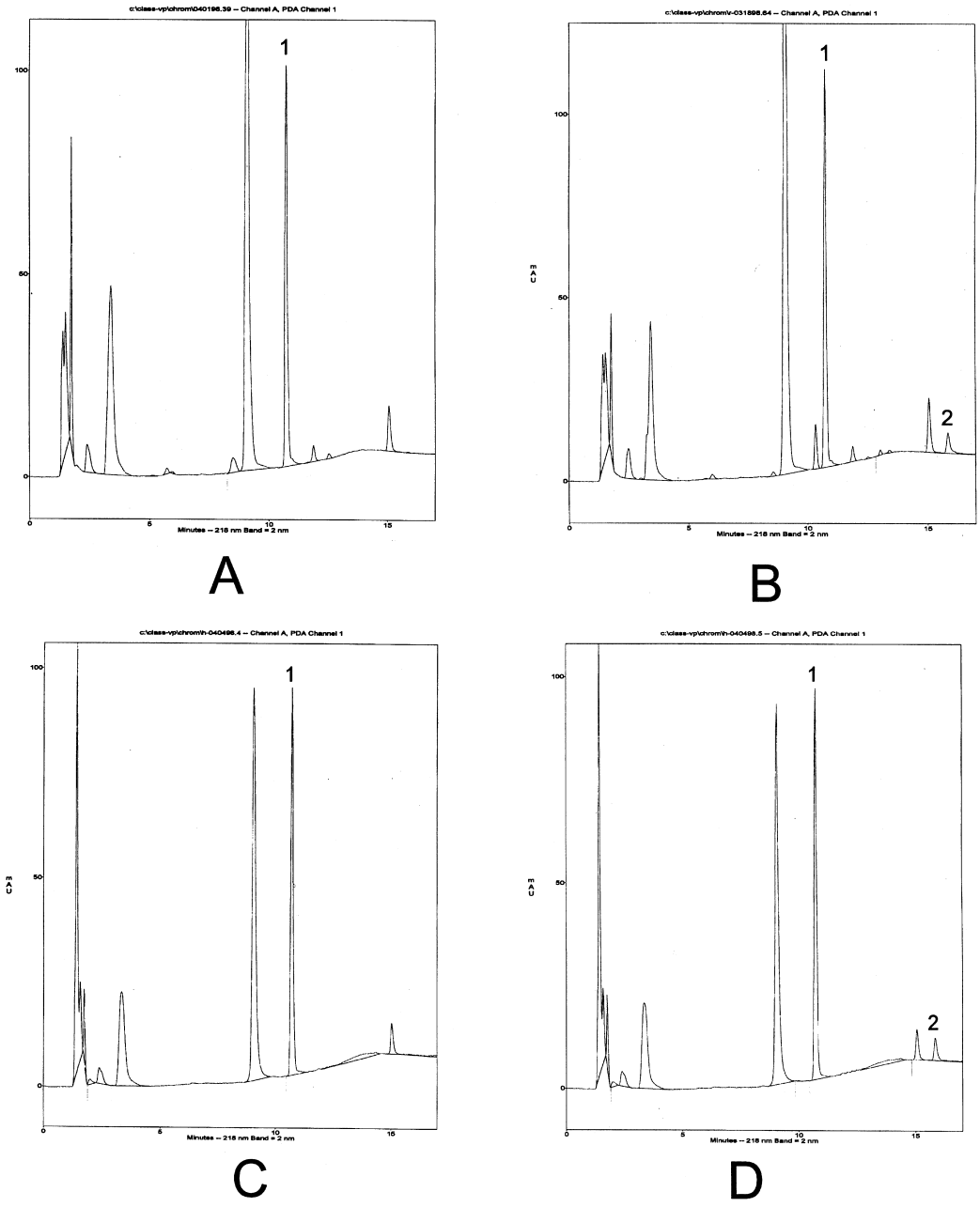

Fig. 1. Representative chromatograms. (A) is a representative chromatogram of a blank (before treatment) rat plasma sample (50 μl), which was run immediately after a plasma sample containing 90 μg/ml MPA to rule out carry-over effects. (B) is a chromatogram of a rat plasma sample (50 μl) taken 36 h after oral administration of 40 mg/kg MPA (calculated concentration: 2.0 μg/ml). (C) is a representative chromatogram of a blank human plasma sample (50 μl). Again, the analysis was run immediately after a plasma sample to which 90 μg/ml MPA was added to exclude potential carry-over effects. (D) is a human plasma sample, to which 2 μg/ml MPA was added. Analytical conditions: sphereclone 5 μm ODS(2), 200×4.6 mm analytical column, mobile phase: acetonitrile–sulfuric acid pH 4.0, gradient: 0 min: 20% acetonitrile, 10 min: 42% acetonitrile followed by a column wash and re-equilibration phase, flow:1.5 ml/min, UV-detection wavelength: 218 nm. In the chromatograms, the internal standard peak with a retention time of 10.6 min is marked (1) and the MPA peak with a retention time of 15.7 min is marked (2).

major advantage of our assay is the fast and simple one-step extraction procedure in combination with a high sensitivity allowing for analysis of 50-μl plasma samples. A laborious multi-step extraction is avoided. MPAG is separated from the IS and can be quantified using this method.

One of our goals was to develop a method with a simple and fast extraction procedure. Naturally, the resulting extracts are not as clean as after more work-intensive and time-consuming multi-step extraction procedures. With our gradient HPLC-method we could easily and completely separate the compounds of interest from potentially interfering material (see Fig. 1). In contrast, isocratic elution did not allow for a reliable separation of MPA and its internal standard from interferences.

According to the recommendations to a recent consensus document [1], plasma is the matrix of choice for the measurement of MPA in patients and was therefore the matrix used in our assay. As demonstrated, our method is also linear and valid for plasma sample volumes less than 50 μl (see partial volume integrity).

Using this method, we have performed several pharmacokinetic and pharmacodynamic studies in rats, analyzing more than 1000 samples.

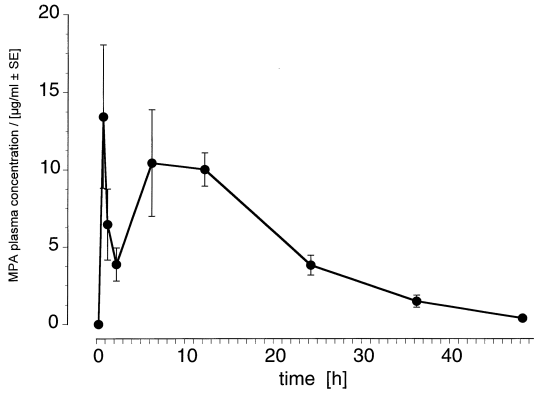

Fig. 2. Forty-eight h MPA pharmacokinetic profile in Lewis rats ($n=6$). Data points represent means±SD ($n=6$).

As an example, a representative mean pharmacokinetic profile of MPA in male adult Lewis rats ($n=6$) after a single oral dose of 40 mg/kg is shown in Fig. 2. The bi-phasic curve can be explained, as known from humans, by a possible enterohepatic circulation of the glucoronidated main MPA metabolite and subsequent conversion back to MPA [1]. Our assay was sensitive enough to measure MPA concentrations above the LLOQ even 48 h after drug administration using a sample volume as small as 50 μl.

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