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Short communication

Simple high performance liquid chromatographic assay for mycophenolic acid in renal transplant patients

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Abstract

A selective and highly accurate HPLC-UV method is described to determine plasma concentrations of mycophenolic acid (MPA), the active metabolite of the prodrugs Cellcept® and Myfortic®. The method is simple and utilizes acidification of plasma and protein precipitation step using a mixture of acetonitrile and phosphate buffer (pH 3). Following vortex mixing and centrifugation, the supernatant (50 μL) was injected onto a Zorbax Eclipse XDB C_{18} column (150 mm × 4.6 mm I.D., 5 μm particle size). A mobile phase composed of acetonitrile and 0.1 M phosphate buffer, pH 3 (43:57) delivered at 1.0 mL/min produced peaks for MPA and the internal standard (Naproxen) in <7 min. Calibration curves were linear ($r^2 > 0.994$) from 1.0–40 μg/mL with intra- and inter-day precision <15% and accuracy >95%. The method's improved sensitivity (LOQ = 1.0 μg/mL) and minimal sample processing allowed rapid monitoring of MPA in human plasma.
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1. Introduction

A basic tenet of clinical pharmacology is that the pharmacologic activity of an exogenously administered agent is related to the free drug concentration available at its receptor or ligand-binding site [1,2]. The study of pharmacokinetic principles is important in the development and practice of rational therapeutics in clinical medicine. This is particularly true in transplant patients because they exhibit heterogeneous immunologic behavior and extremely variable toxic threshold of the immunosuppressive medications. In addition, they display considerable variability in pharmacokinetic behavior [3,4]. Utilizing the pharmacokinetic (PK) principles and dose-concentration relationships are central to therapeutic drug monitoring in optimizing drug dosing to target concentrations [3,5]. Such research requires analytical methods that are both sensitive and simple due to the high number of samples that require processing.

Mycophenolic acid (MPA, Fig. 1) derivatives (Cellcept® or Myfortic®) are currently the immunosuppressive agent of choice in renal transplantation in combination with a calcineurin inhibitor and steroids [6]. Their introduction in clinical medicine represented a significant advance because they improve graft survival rates. Optimal use of these new immunosuppressive drugs requires knowledge of their pharmacobiology. For example, presystemic hydrolysis of Cellcept® and Myfortic® by systemic esterases release MPA, the active compound [2]. MPA is bound to albumin. The ratio between the free and bound components is affected by a variety of conditions including hypoalbuminemia, renal insufficiency and concomitant cyclosporin, tacrolimus or steroid therapy [2,7,8]. These variables are commonly present in transplant patients, a situation that argues for monitoring of MPA level.

There are more evidences to suggest that the measurement of MPA plasma concentrations may add to patient management. The inter-individual variability in PK, the changes in PK over time, the influence of co-medication and the correlations between drug concentrations and outcome are arguments in favor of MPA monitoring [9]. Monitoring MPA plasma concentrations is thought to be useful particularly within the first 2 months after transplantation, prior to measured changes in other immunosuppressive doses such as steroid withdrawal and at the time of measure clinical events such as rejection or infection

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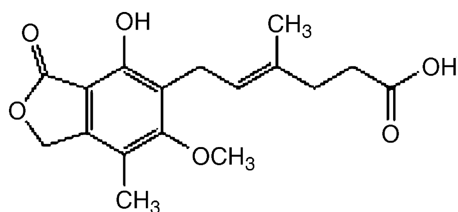

Fig. 1. Chemical structure of MPA.

[4]. As such, some centers have implemented MPA monitoring schedules into their transplant programs. To implement MPA monitoring in routine clinical practice, the availability of a simplified, sensitive and reproducible method is desirable.

Several HPLC methods with UV detection have been developed for the determination of MPA in human plasma [10–19]. Some of these methods permit quantitation of MPA and its glucuronide metabolite [14–17]. Those methods require the use of gradient elution system [11,13,19], use of more than one stationary phase [16] or application of tedious sample extraction procedures [11,14]. More sensitive methods for MPA assay in plasma and other biological fluids have been developed, but they used liquid chromatography with tandem mass spectrometry [20,21].

In this paper, we describe the development, optimization and validation of simple, rapid, and sensitive HPLC-UV detection method for the assay of MPA in human plasma. Furthermore, we demonstrate the application of this method to quantify MPA levels in renal transplant patient following the oral administration of one single dose of 1.5 g Cellcept. The distinct advantages of this method over other reported methods include its simplicity, and use of inexpensive and readily available internal standard (Naproxen, NAP) to increase the method's reproducibility. The study protocol was approved by the University of Saskatchewan ethical board.

2. Experimental

2.1. Chemicals and reagents

MPA was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Naproxen was purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). HPLC grade acetonitrile and methanol were purchased from EMD chemicals (Darmstadt, Germany). A Milli Q Synthesis (Millipore, Bedford, MA) water purification system provided purified deionized water. All other chemicals used were analytical grade.

2.2. Apparatus and HPLC conditions

The HPLC system consisted of Waters model 2695 Alliance separation module, model 2996 photodiode array detector and Empower data module (Millipore-Waters, Milford, MA, USA). Absorbance was monitored at 215 nm (λ_{max} for MPA). Chromatographic separation was carried out on Zorbax Eclipse XDB C_{18} column (150 mm × 4.6 mm I.D., 5 μm particle size). The column was kept at 25 °C. The analytes were eluted under isocratic conditions using a mobile phase composed of acetonitrile and 0.1 M phosphate buffer, pH 3 (43:57) delivered at 1.0 mL/min.

2.3. Preparation of stock and working solutions

Stock solutions of MPA (1000 μg/mL) and naproxen (100 μg/mL) were prepared in methanol and stored at −20 °C. Working solutions of MPA (10–400 μg/mL) were prepared by serial dilutions of the 1000 μg/mL stock solution with methanol. These solutions were stored at 4 °C protected from light and were stable for at least 1 week.

2.4. Preparation of calibration standards and quality control (QC) samples

Known amounts of working solutions were diluted with drug-free human plasma to achieve calibration standards of 1.0–40 μg/mL MPA. Three quality control (QC) samples at 3.0 μg/mL (low), 10 μg/mL (medium) and 20 μg/mL (high) were prepared independent of those used for the calibration curves. These QC samples were prepared on the day of analysis in the same way as calibration standards.

2.5. Sample preparation

To 200 μL calibration standards, QC samples, or patient plasma samples, 25 μL of the internal standard solution (10 μg/mL in methanol) and 500 μL of a mixture of 1 M phosphate buffer, pH 3.0 and acetonitrile (20:80) were added. The mixtures were vortex mixed for 20 s. After centrifugation at 12 000 × g in an eppendorf microcentrifuge tubes for 5 min, 50 μL of the supernatant was injected directly onto the analytical column for immediate HPLC analysis.

2.6. Method validation

Method validation procedures were performed according to FDA guidelines (www.fda.gov/cder/guidance/cmc3.pdf) to evaluate the suitability of the method for the quantitative determination of MPA in human plasma. Specificity was tested by analysis of four independent drug-free human plasma samples supplemented only with internal standard to ensure the absence of endogenous compounds with the same retention time as MPA.

The linearity of the method was evaluated by processing a six-point calibration curve range from 1.0 to 40 μg/mL on five different days. The peak height ratios between MPA and the internal standard were plotted against the nominal concentration of MPA. A linear least-squares regression analysis was conducted to determine slope, intercept and coefficient of determination (r^2) to demonstrate linearity of the method.

The accuracy and precision of the proposed method were determined by analysis of the QC samples. The intra-day accuracy and precision were assessed from the results of six replicate analyses of QC samples (3, 10 and 20 μg/mL) on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on six consecutive days. Preci-

sion is expressed as % relative standard deviation (R.S.D.), while accuracy (%) is expressed as [(calculated amount/predicted amount) × 100].

The low limit of detection (LLOD) was defined as the lowest detectable concentration, taking into consideration a signal-to-noise ratio of 3 [22]. Low limit of quantification (LLOQ) was determined at the lowest concentration at which the precision, expressed as % R.S.D., is less than 20% and accuracy, expressed as relative difference between the measured and true value, is less than 20% [22].

The recovery of MPA was determined by comparison of peak heights obtained from injection of 50 μL aliquots of either MPA standard (1.0, 5 and 10 μg/mL) prepared in methanol or samples containing the same amount of MPA after spiking in blank human plasma ($n = 6$) and processed as indicated above.

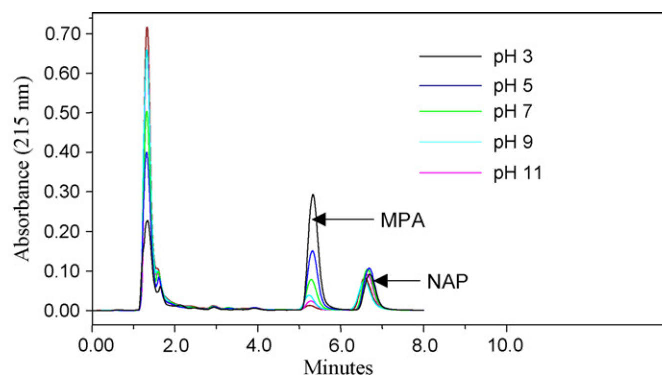

Fig. 2. Effect of buffer pH on the recovery of MPA and NAP from human plasma.

3. Results and discussion

3.1. Method optimization

We could not demonstrate any MPA peak when we followed a protocol of a previously published study where sample acidification was not performed. Therefore, we had to change the mobile phase composition, stationary phase and finally the extraction procedures. An interesting finding was the correlation between the pH of the buffer used to precipitate the proteins and the recovered amount of MPA and the internal standard. Fig. 2 illustrates typical overlaid chromatograms of blank human plasma spiked with 5 μg/mL MPA and 10 μg/mL NAP and sample extraction was performed using buffers with different pH. As the chromatograms show, the higher the acidity of the buffer used in the extraction, the higher is the MPA recovery. The chromatogram shown in Fig. 3A demonstrates that no endogenous peaks in

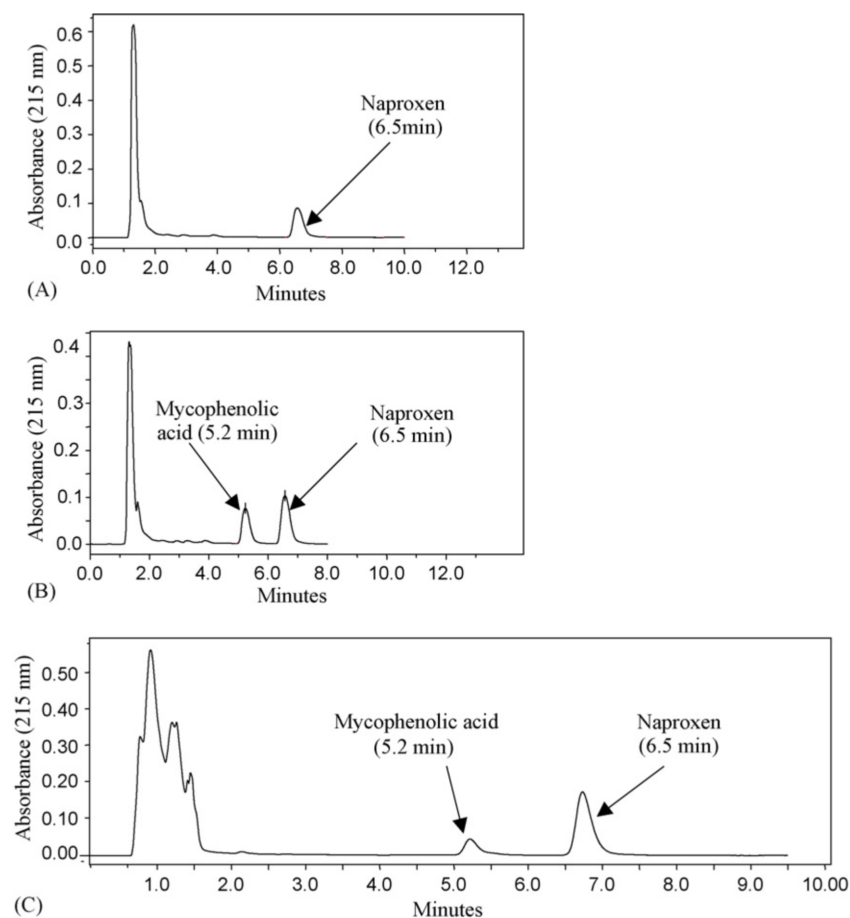

Fig. 3. HPLC chromatograms: (A) blank human plasma sample supplemented only with the internal standard; (B) working solution containing MPA (3 μg/mL) and internal standard; (C) patient plasma sample after 3 h of MPA oral administration (1.5 g Cellcept®).

Table 1
Linearity data for the determination of MPA with the proposed HPLC method

Calibration curve	Slope	Intercept	R^2
1	0.0782	0.0143	0.993
2	0.0783	0.0079	0.994
3	0.0786	0.0082	0.999
4	0.0777	0.0245	0.996
5	0.0865	0.0127	0.999
Mean	0.07986	0.01352	0.9962
S.D.	0.00372	0.00674	0.0027

S.D.: standard deviation of the mean.

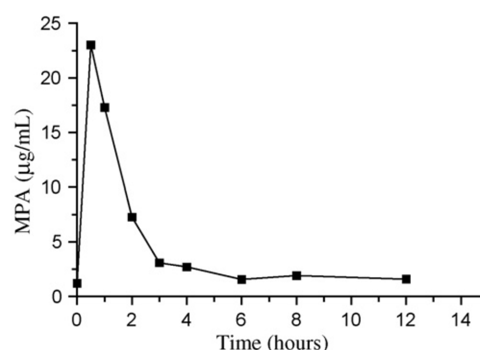

Fig. 4. MPA plasma concentration time profile in a renal transplant patient following oral administration of (1.5 g Cellcept®).

human plasma are co-eluted with the MPA or the internal standard, which indicates that the method is specific for measuring MPA in human plasma samples. Figs. 3B and C indicate that under the chromatographic conditions used for the analysis, the retention times for MPA and NAP were 5.2 and 6.5 min, respectively.

3.2. Method validation

MPA limit of detection (LLOD) was 0.25 μg/mL and the limit of quantitation (LLOQ) was 1.0 μg/mL. The LLOQ was calculated as the lowest MPA concentration in the working solutions that could be measured routinely with acceptable accuracy (90–110%) and precision (R.S.D. < 20%).

The method was linear over MPA concentration range of 1.0–40 μg/mL. Coefficients of determination were greater than 0.994 and the relevant slope values were statistically different from zero ($p < 0.001$) (Table 1). Although intercepts of the calibration curves were significantly different from zero (Table 1), they did not affect the accuracy of the method. Furthermore, a linear regression of the back-calculated concentrations versus the nominal concentrations produced a slope of unity and an intercept equal to zero.

Table 2 summarizes the results for intra- and inter-day accuracy and precision. During the course of method validation, intra-day and inter-day precision was less than 10%. The accuracy of estimated MPA concentrations ranged from 92–106%. These data show that our method is both accurate and precise in human plasma samples. The mean ± S.D. absolute recoveries of MPA at 3, 10 and 20 μg/mL were 102 ± 3%, 96 ± 3% and 97 ± 2%, respectively. The mean ± S.D. absolute recovery of the internal standard was 95 ± 2%. Our method, which involved only a protein precipitation step and no extraction procedures, provided good specificity and analytical recoveries.

3.3. Method application

The simple sample processing procedures led to improved analytical sensitivity, which allowed the measurement of MPA in plasma of a renal transplant patient. Fig. 4 shows a plasma concentration-time profile of MPA in a renal transplant patient following oral administration of 1.5 g Cellcept®. Maximum plasma concentration of 23 μg/mL was achieved within 1 h after drug administration. Plasma concentration of MPA remained relatively constant (approximately 2.5 μg/mL) between 2.5 h and 12 h postdosing. Suitability of our method to monitor MPA plasma concentration with knowledge of MPA therapeutic range, can help as a tool to adjust dosing regimen in renal transplant patients so that highest therapeutic effect can be achieved without potential risk of toxicity.

The total analytical run time was less than 7 min, which allowed sequential assay of multiple samples in a relatively short time period. The minimal sample preparation and short analytical run times offer an economical advantage to currently available methods [10–21] with respect to resources and operator time.

Table 2
Intra-day ($n = 6$) and inter-day (6 consecutive days) accuracy and precision values of MPA determination by HPLC-UV detection in human plasma

Quality controls (QC)	Nominal concentration (μg/mL)	Observed concentration (mean ± S.D., μg/mL)	Accuracy (%)	Precision (CV%)
Intra-day accuracy and precision				
QC1	3	3.1 ± 0.1	102	4.3
QC2	10	9.7 ± 0.25	96	2.57
QC3	20	19.5 ± 0.44	97	2.25
Inter-day accuracy and precision				
QC1	3	3.19 ± 0.23	106	7.23
QC2	10	9.4 ± 0.44	94	4.68
QC3	20	18.45 ± 0.798	92.3	4.33

4. Conclusion

In this study we report a simple, rapid, and accurate HPLC method for the quantification of MPA in a renal transplant patient to serve as a marker to monitor drug level. The proposed liquid chromatographic with UV detection method is selective, sensitive with a detection limit of 0.25 μg/mL. In addition, this method is accurate and precise for the quantification of MPA in blood of transplant patients in the range of 1–40 μg/mL.

Acknowledgements

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