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Quantification of free mycophenolic acid by high-performance liquid chromatography–atmospheric pressure chemical ionisation tandem mass spectrometry

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Abstract

To facilitate the investigation of free mycophenolic acid concentrations we developed a high-performance liquid chromatography tandem mass spectrometry method using indomethacin as an internal standard. Free drug was isolated from plasma samples (500 μl) using ultrafiltration. The analytes were extracted from the ultrafiltrate (200 μl) using C_{18} solid-phase extraction. Detection was by selected reactant monitoring of mycophenolic acid (m/z 318.9→190.9) and the internal standard (m/z 356.0→297.1) with an atmospheric pressure chemical ionisation interface. The total chromatographic analysis time was 12 min. The method was found to be linear over the range investigated, 2.5–200 μg/l (r>0.990, n=6). The relative recovery of the method for the control samples studied (7.5, 40.0 and 150 μg/l) ranged from 95 to 104%. The imprecision of the method, expressed in terms of intra- and inter-day coefficients of variation, was <8 and <9%, respectively. Further, analysis of pooled patient plasma produced an intra-day imprecision of 6.6%. The signal-to-noise ratio at the limit of quantification (2.5 μg/l) was approximately 5:1. The mean absolute recovery (n=6) of mycophenolic acid and the internal standard were 76.0±13.5% and 86.0±9.1%, respectively. The method reported provides an accurate and precise quantification of free mycophenolic acid over a wide analytical range and thus can be used for routine monitoring and pharmacokinetic studies.

Keywords: Mycophenolic acid

1. Introduction

Mycophenolate mofetil (CellCept®, Fig. 1A) is an immunosuppressant prodrug registered for the prophylaxis of acute rejection in renal transplant recipients. Mycophenolate mofetil is administered orally and is rapidly hydrolysed to the active immunosuppressive agent, mycophenolic acid (Fig. 1B). Mycophenolic acid is a potent, noncompetitive and reversible inhibitor of inosine monophosphate dehydrogenase. Through its mode of action, lymphocyte purine synthesis and thus proliferation is inhibited [1,2]. Mycophenolic acid undergoes glucuronidation (>80%) with the pharmacologically inactive gluc-

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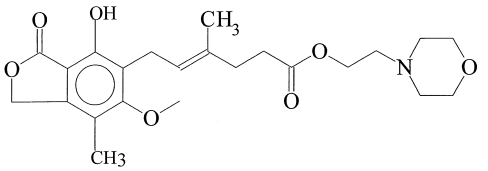
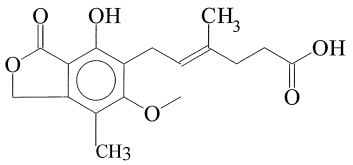
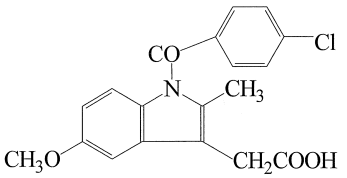


(A) Mycophenolate mofetil



(B) Mycophenolic acid



(C) Indomethacin (internal standard)

Fig. 1. The chemical structures of (A) mycophenolate mofetil, (B) mycophenolic acid, and (C) indomethacin (internal standard).

uronide being excreted into the urine [3,4]. The pharmacokinetics of mycophenolic acid has been shown to have wide inter-patient variability [5,6] and to change with time post-transplant [4].

In vitro studies, by Nowak and Shaw [7] showed that mycophenolic acid is extensively and avidly bound to human serum albumin. Only the free drug is pharmacologically active. The clinical relevance of monitoring of free mycophenolic acid is yet to be established, but changes in plasma protein binding due to such disease states as liver disease, uremia or hypoalbuminemia could potentially lead to an altered efficacy or toxicity profile. In a study on mycophenolic acid pharmacokinetics in pediatric and adult renal transplants, Weber et al. [8] reported that renal impairment and decreased serum albumin concentrations, led not only to an increase in free fraction of mycophenolic acid but also an increase in free mycophenolic acid concentration.

Several techniques are available for the isolation of a free drug fraction, namely microdialysis, equilibrium dialysis and ultrafiltration [9]. It has been shown that ultrafiltration and equilibrium dialysis produced comparable results for the isolation of mycophenolic acid free fraction [7]. Ultrafiltration has the greatest clinical utility as it is rapid and relatively simple compared with other techniques [10]. High-performance liquid chromatography with ultra-violet detection (HPLC–UV) has been reported for measuring total mycophenolic acid concentration using solid-phase extraction [11,12]. Shipkova et al. [13] reported the utilization of ultrafiltration coupled with HPLC–UV to determine free mycophenolic acid concentrations.

In this study, we report the use of ultrafiltration followed by solid-phase extraction for sample preparation and HPLC–tandem mass spectrometry detection for the quantification of free mycophenolic acid in human plasma.

2. Experimental

2.1. Materials

Mycophenolic acid was obtained from F. Hoffmann–La Roche Ltd. (Basel, Switzerland). Indomethacin (internal standard, Fig. 1C) was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). A working stock of internal standard (250 μg/l) was prepared in methanol. All solvents were HPLC grade and all reagents analytical-reagent grade. HPLC quality water was prepared using a Millipore Milli-Q purification system (Millipore, Milford, MA, USA). Centrifree Micropartition System consisting of a 1 ml reservoir and a 30 000 Da MW cutoff membrane were used for ultrafiltration (Amicon, Danvers, MA, USA). Calibration standards (2.5, 5.0, 10.0, 25.0, 50.0, 100, and 200 μg/l) and quality control samples (7.5, 40.0 and 150 μg/l) were prepared in sodium chloride solution (9 g/l, pH 7.4). A further quality control sample was prepared by pooling plasma from 6 cardiac transplant recipients who were receiving mycophenolate mofetil therapy.

2.2. HPLC mass spectrometry apparatus and conditions

The HPLC system consisted of a 616 pump with a 600S controller, column oven with temperature control module (Waters, Milford, MA, USA) and an IS200 autosampler (Perkin-Elmer, Danbury, CT, USA). The HPLC column was a Novapak C_{18} column (150×2.1 mm I.D., 4 μm, Waters), maintained at a temperature of 35°C. The mobile phase consisted of 55% methanol:45% ammonium formate buffer (2 m*M*, pH 3.8). The flow-rate employed was 0.5 ml/min.

Mass spectrometric detection was performed on an API III triple quadrupole instrument (PE-Sciex, Thornhill, Toronto, Canada) using selected reactant monitoring of mycophenolic acid (*m/z* 318.9→190.9) and internal standard (*m/z* 356.0→297.1). The collision energy for collision induced fragmentation was −21.3 V. The atmospheric pressure chemical ionisation interface was operated at 500°C in negative ionisation mode. The discharge current and orifice potential were set to −2.5 μA and −40 V, respectively. The nebulizer and auxilary gas flows were maintained at 0.6 and 2.0 l/min, respectively. Data was acquired with a dwell time of 300 ms, a pause time of 100 ms and a scan rate of 0.83/s. The collision-induced mass spectra obtained under these mass spectrometric conditions for mycophenolic acid and the internal standard are shown in Fig. 2.

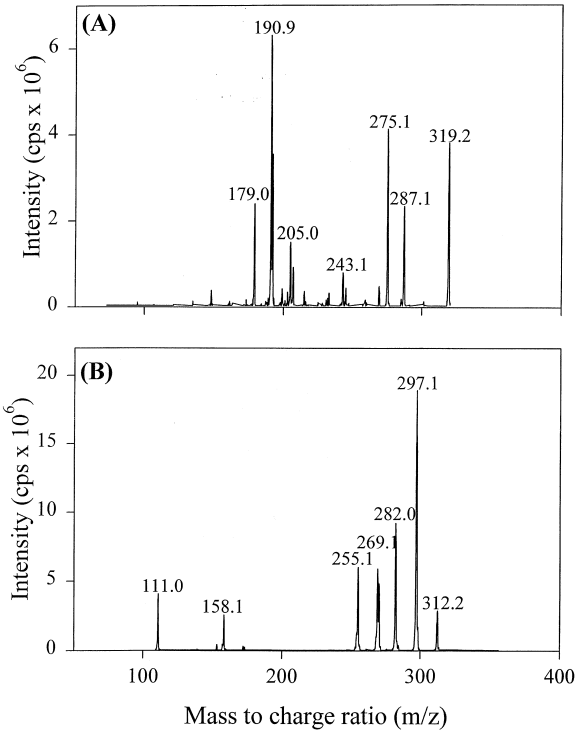


Fig. 2. The collision-induced mass spectra of (A) mycophenolic acid and (B) the internal standard. The major product ions *m/z* 190.9 and *m/z* 297.1 were used for selected reactant monitoring of mycophenolic acid and the internal standard, respectively.

2.3. Sample preparation

Ultrafiltration conditions were based on the method described by Nowak and Shaw [7]. Plasma samples (500 μl) were placed in sealed ultrafiltration tubes and centrifuged at 3000 *g* in a Beckman fixed rotor centrifuge (20 min, 20°C). The ultrafiltrate, standard and control samples (200 μl) were added to internal standard working stock (100 μl) and 0.05 *M* hydrochloric acid (500 μl) in glass culture tubes. Samples were vortex mixed (1 min) and centrifuged (1 min, 850 *g*). The mixtures were applied to 100-mg C_{18} solid-phase extraction cartridges (Waters) which had been preconditioned with methanol (2 ml) and water (2 ml). The loaded cartridges were washed sequentially with water (1 ml), and 50% methanol/0.02 *M* ammonium formate buffer, pH 3.0 (1 ml). The washed cartridges were placed under a full vacuum for 15 min. The analytes were eluted with methanol (1 ml) and the solvent removed under a stream of air (45°C). The residue was dissolved in mobile phase (50 μl). An aliquot of the mixture (20 μl) was injected on column.

2.4. Assay validation studies

Linearity was tested by analysing calibration standards containing known (weighed-in) amounts of mycophenolic acid over a concentration range (2.5–200 μg/l, $n=6$). A weighted linear regression model ($1/x^2$) was used throughout the study as recommended for calibration curves that span a wide

concentration range [14]. The specificity of the method was evaluated by analysing 10 plasma samples from different transplant recipients not receiving mycophenolate mofetil therapy. The relative recovery and inter-day precision were determined from the back-calculated results of the linearity study ($n=6$). The relative recovery and inter-day precision were determined by assaying quality control samples, prepared in saline, in singlicate on each of 5 days. The intra-day precision was determined by assaying quality control samples prepared in saline and a pooled patient sample (see Materials section) in replicates of 5 on one day. The relative recovery was expressed as the mean assayed result for the quality control samples ($n=5$) as a percentage of the weighed-in concentration. Absolute recoveries of the analytes were determined by comparing the peak areas of extracted ultrafiltrate samples, from 6 different subjects, spiked with mycophenolic acid and the internal standard before and after solid-phase extraction.

3. Results

The chromatographic conditions utilized in this assay achieved retention times of 2.8 min for mycophenolic acid and 8.9 min for the internal standard. The total chromatographic analysis time was 12 min. Analysis of 10 blood samples from transplant recipients not receiving mycophenolic acid therapy revealed no interference at the retention time of the two analytes. A representative chromatogram of the specificity study is shown in Fig. 3(A). A typical chromatogram of a calibration standard (5.0 μg/l) and a blood sample obtained from a renal transplant recipient receiving oral mycophenolate mofetil (18.6 μg/l) are shown in Fig. 3.

The method was found to be linear over the range investigated, 2.5–200 μg/l ($r>0.990$, $n=6$). The relative recovery and inter-day imprecision of the method, determined over the calibration range, was between 97 and 103% and <15%, respectively. From these performance data, we defined the lower limit of quantification as the lowest calibration standard (2.5 μg/l) and the upper limit of quantification as the highest standard (200 μg/l). The methods

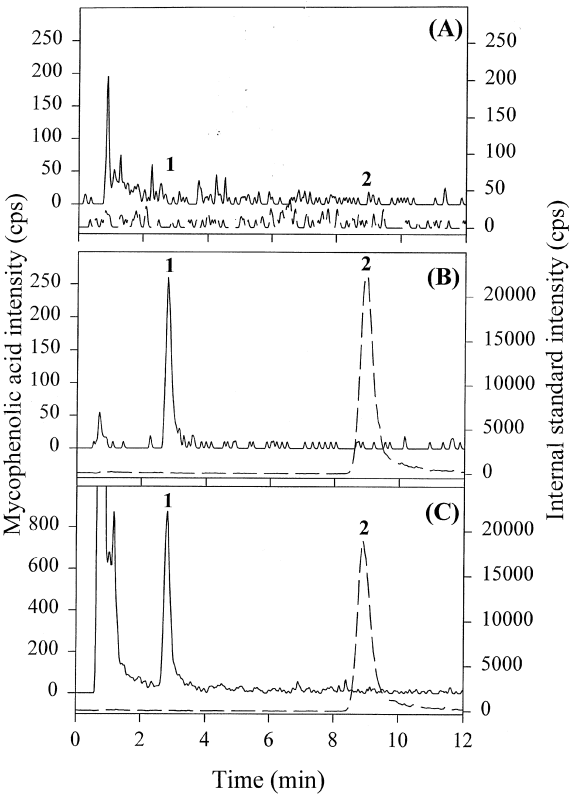


Fig. 3. Representative chromatograms of (A) blank plasma, (B) calibration standard (5.0 μg/l), and (C) a patient sample (18.6 μg/l). The solid line represents mycophenolic acid (m/z 318.9→190.9) and the dashed line the internal standard (m/z 356.0→297.1). The numbers 1 and 2 show the retention time of mycophenolic acid and the internal standard, respectively.

analytical performance at the limits of quantification was deemed acceptable under the guidelines of Shah et al. [15]. The signal-to-noise ratio at the limit of quantification was approximately 5:1.

The relative recovery of the method for the control samples studied (7.5, 40.0 and 150 μg/l) ranged from 95 to 104% (Table 1). The imprecision of the method, expressed in terms of intra- and inter-day coefficients of variation, was <8% and <9%, respectively (Table 1). Further, analysis of pooled patient plasma produced an intra-day imprecision of 6.6%. The mean absolute recovery ($n=6$) of mycophenolic acid and the internal standard were

Table 1
The relative recovery and imprecision of the HPLC mass spectrometry method

MPA concentration (μg/l)	Mean (μg/l)	Relative recovery (%)	Intra-day C.V. (%)	Inter-day C.V. (%)
Quality control (7.5)	7.13	95.1	7.8	8.8
Quality control (40)	40.1	100	7.5	5.8
Quality control (150)	156	104	3.5	4.0
Pooled plasma	90.8	***	6.6	***

*** Not determined.

determined to be 76.0±13.5% and 86.0±9.1%, respectively.

A 10-h pharmacokinetic study of free and total mycophenolic acid, obtained from a renal transplant recipient who received chronic oral dosing of mycophenolate mofetil (1 g Bid), is shown in Fig. 4. Total mycophenolic acid was determined by our previously reported HPLC–UV method [12]. The time to achieve maximum free mycophenolic acid concentration was rapid (2.0 h) with a maximum concentration of 122.5 μg/l and the area under the concentration time curve was 409.2 μg.h/l. The mycophenolic acid free fraction for this patient, determined by the ratio of free to total mycophenolic area under the curve, was 1.2%.

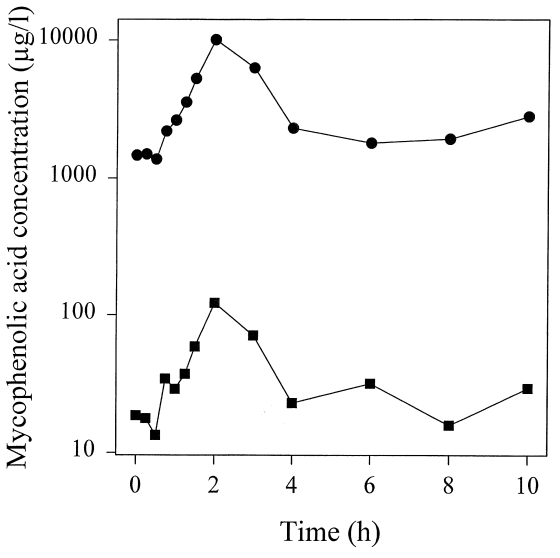


Fig. 4. A 10-h pharmacokinetic profile of free (squares) and total (circles) mycophenolic acid, obtained from a renal transplant recipient who received chronic oral dosing of mycophenolate mofetil (1 g Bid). Total mycophenolic acid was determined by our previously reported HPLC–UV method [12].

4. Discussion and conclusions

We have previously reported an assay for the determination of total mycophenolic acid in plasma by HPLC–UV [12]. In the development of a free mycophenolic acid method, the lower limit of quantification of the total concentration method (100 μg/l) was found to be unsuitable. We have previously utilized HPLC mass spectrometry for the determination of low μg/l concentrations of xenobiotics and endogenous compounds [16–18]. Mass spectrometric detection was evaluated and found to provide sufficient sensitivity to measure trough free mycophenolic acid concentrations (5–10 μg/l). The limit of quantification of this method (2.5 μg/l) compares favourably with the HPLC–UV method (10 μg/l) of Shipkova et al. [13].

The clinical usefulness of this method has been demonstrated with the concentration time profile of a single renal transplant patient receiving chronic oral dosing of mycophenolate mofetil (Fig. 4). For this patient, the method was capable of measuring all samples throughout the profile including trough and peak concentrations. The free mycophenolic acid pharmacokinetic parameters obtained from this study are in agreement with those reported by Weber et al. [8]. Further, the technical simplicity of ultrafiltration to isolate the free drug makes this method suitable for routine clinical use.

In conclusion, the validated method described provides the first reported use of mass spectrometry for the quantification of free mycophenolic acid. The accurate and precise quantification of free mycophenolic acid over the wide analytical range 2.5–200 μg/l will facilitate the investigation of free mycophenolic acid pharmacokinetics.

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