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Simultaneous determination of free mycophenolic acid and its glucuronide in serum of patients under mycophenolate mophetil therapy by ion-pair reversed-phase liquid chromatography with diode array UV detection

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Abstract

An high performance liquid chromatography (HPLC)–UV method for the simultaneous determination of the free forms of mycophenolic acid (MPA) and its phenol glucuronide (MPAG) in human serum samples was developed for the first time.

Chromatographic separation was performed on octadecylsilane based stationary phase in combination with a mobile phase of methanol/buffered tetrabutylammonium (TBA) salt mixture.

Sample pretreatment consisted of an ultrafiltration step followed by clean-up/enrichment on a C_{18} solid-phase extraction (SPE) cartridge. Average recoveries of (99.7 ± 0.2)% and (64.1 ± 6.9)% for free MPA and MPAG, respectively, were estimated in the concentration range from 0.5 to 10 μg/ml. The within-day and between-days coefficients of variation were 0.4 and 0.8% for free MPA (0.1 μg/ml spiking level) and 0.8 and 1.6% for free MPAG (5 μg/ml spiking level), respectively. The linear ranges for free MPA and MPAG were 0.06–1 and 0.2–10 μg/ml, respectively. Detection limits of 4 and 17 ng/ml for free MPA and MPAG were estimated in spiked serum.

The same HPLC method was also capable of a simultaneous determination of the total concentration of MPA and MPAG when coupled to a proper sample pretreatment step. The potential of the method is demonstrated by excretion kinetics measurement in serum of patients receiving MMF therapy.

Keywords: Mycophenolic acid; Mycophenolic acid glucuronide; Mycophenolate mophetil

1. Introduction

Mycophenolic acid (MPA) is an immunosuppressive agent acting as a selective, reversible, non competitive inhibitor of *inosine monophosphate dehydrogenase* (IMPDH), a key enzyme involved in the de novo synthesis of the purine nucleotide, guanosine monophosphate. Due to this peculiar action of MPA, the intracellular guanine nucleotide pool decreases significantly resulting in an inhibition of DNA synthesis. Since lymphocytes rely on the de novo purine biosynthesis more than on the purine salvage pathway (catalyzed by *hypoxanthine–guanine phosphoribosyl transferase*) MPA prevents proliferation of both T and B lymphocytes [1]. Indeed MPA has been shown to be very effective in preventing acute rejection in patients receiving organ transplants [2–5].

A prodrug of MPA, the morpholinoethyl ester mycophenolate mophetil (MMF) has been synthesized to increase the bioavailability of mycophenolic acid. After oral administration, the ester is rapidly and completely adsorbed and hydrolyzed to its active metabolite (MPA) by blood esterases. MPA is metabolized by a liver *UDP-glucuronosyl transferase*, to its pharmacologically inactive phenol glucuronide (mycophenolic acid glucuronide, MPAG), which is primarily

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excreted in urine. However, an enterohepatic pathway involving MPA passage into the gastrointestinal tract via biliary excretion, hydrolysis to MPA (via *β-glucuronidase* in gut flora) and reabsorption of the latter into the systemic circulation, can contribute to blood concentration of the active drug.

Other factors, which intervene in an individual at different times or vary between individuals, can alter the amount of the bio-available drug. It is known that MPA levels are influenced by binding to proteins, mainly human serum albumin (HSA). Since only the free drug is pharmacologically active this need to be measured, in addition to the total MPA concentration, in order to calculate the free fraction. Significant increases of MPA free concentration and/or MPA free fraction have been observed in patient with severe renal dysfunction causing hypoalbuminemia status; similar effects could also be observed with increasing concentration of MPAG due to a competition for HSA binding sites.

These observations, together with the unavoidable interpatient MPA pharmacokinetics and the possible enterohepatic pathway for MPAG/MPA, suggest that the MMF dosage regimen should be tailored on individual (or individual groups) bases, in order to achieve an appropriate level of the active drug, minimizing MMF side effects.

To this purpose, a method for the simultaneous determination of blood concentrations of MPA and MPAG (free form plus protein bound) is highly advisable. Several high performance liquid chromatography (HPLC) methods [6–21] are today available for the determination of total MPA and/or MPAG concentrations in biological fluids. Since blood concentrations in the low μg/ml level need to be determined, the analytical demand in term of sample treatment, sensitivity, selectivity and chromatographic efficiency is not particularly stringent. Then, the most common approaches consist in simple deproteinization step followed by ion suppressed reverse phase HPLC with UV detection [6,7] or ion-pair HPLC with UV [8,9] or fluorescence detection [10]. Reverse phase HPLC with post column addition of 2 M NaOH and fluorescence detection of deprotonated (phenolate anion) analyte has been described by Renner et al. [11]. A detection limit (S/N = 3) of 3 ng/ml was claimed for a 100 μl plasma sample subjected to a deproteinization step. MMF could be simultaneously determined while the phenolglucuronide MPAG, not directly accessible for fluorescence detection, was separately analysed after enzymatic deglucuronidation to MPA.

Determination of free MPA and MPAG is a more challenging problem both in term of sample treatment and limit of detection required (low ng/ml). Willis et al. [12] used HPLC with atmospheric pressure chemical ionization tandem mass spectrometry for free MPA determination in plasma samples. The lowest detectable concentration, at S/N = 5, was 2.5 ng/ml for a 500 μl of plasma subjected to ultrafiltration and preconcentration by solid-phase extraction (SPE).

Free MPAG measurement by HPLC–UV has been very recently proposed by Atcheson et al. [13]. However, a simultaneous determination of free MPA and MPAG was not possible and free MPA was measured in a separate assay by HPLC with electrospray tandem mass spectrometry.

The present work describes the first HPLC–UV detection method capable of simultaneous determination of free MPA and MPAG in serum samples. An octadecylsilane based stationary phase is used in combination with a mobile phase of methanol/buffered tetrabutylammonium (TBA) salt mixture. The eluent composition was properly optimized in order to achieve the highest possible chromatographic efficiency. The same HPLC method allows also the simultaneous determination of the total concentration of MPA and MPAG by simply changing the sample pretreatment step. The potential of the method is demonstrated by excretion kinetics measurement in serum of patients receiving MMF therapy.

2. Experimental

2.1. Chemicals

MPA (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phtalanyl)-4-methyl-4-hexenoic acid) and its phenyl glucuronide MPAG (98%) were kindly donated by Hoffmann-La Roche AG (Basel, Switzerland).

Stock solutions (1 mg/ml) of MPA and MPAG were prepared in methanol and stored at 4 °C in the dark. Dilute solutions were prepared just before use in triply distilled water. Organic solvents (Carlo Erba, Milan, Italy), were HPLC grade. Mobile phase was filtered through a 0.45 μm membrane (Whatman Limited, Maidstone, UK) before use.

2.2. Apparatus

The HPLC system consisted of a Spectra System Pump model P2000 (ThermoQuest, San Jose, CA, USA) equipped with a Rheodyne 7125 injection valve (20 μl injection loop), a Supelcosil LC-18-DB column (250 mm × 2.1 mm i.d., 5 μm packing, Supelco) and a photodiode-array detector (Spectra System model UV6000LP) controlled by ChromQuest software running on a personal computer.

The ultrafiltration system comprises a Centricon YM-50 centrifugal filter device (Millipore Corporation, Bedford, MA, USA) equipped with a regenerated cellulose membrane (30 kDa cut-off) and an AIC 4222 centrifuge (Cecchinato, Mestre, Italy) operated at 5.000 × *g*. The centrifugal filter device consisted of a sample reservoir containing the membrane, a retentate vial and a filtrate vial. The centrifugal filter devices were pre-rinsed before use as specified in the manual accompanying the product. Solid-phase extraction (SPE) was performed onto Supelclean solid-phase extraction tubes (Supelco) containing 100 mg of a 40 μm particle size C_{18} packing. The SPE tube was preconditioned eluting in sequence: 2 ml MeOH, 2 ml water and 2 ml of a MeOH/phosphate buffer (5 mM, pH 7) mixture (20:80, v/v) containing 5 mM TBABr.

2.3. Chromatographic and detection conditions

The mobile phase used was a methanol/phosphate buffer (5 mM, pH 7) mixture (50:50, v/v) containing 5 mM TBABr as ion-pairing agent. The flow rate was 0.2 ml min^{-1} and temperature was ambient. Mobile phase was degassed on-line by an SCM1000 Vacuum Membrane Degasser (Thermo Separation Products). Detection wavelength was 254 nm (5 nm band-width). Spectra were acquired in the 230–380 nm range (5 nm band-width). Peak purity could be checked by the technique of spectra overlaying, after normalization.

2.4. Human serum samples

Drug free serum samples were collected from healthy volunteers. Two patients under MMF therapy (l g dose administered every 12 h) have been considered in this study. One of these (patient A) was affected by glomerulonephritis; the other (patient B) was kidney transplanted. Blood samples were collected immediately before dose administration (t = 0) and then after 1, 2, 4 and 6 h.

2.5. Determination of free fractions of MPA and MPAG

A 500 μl serum aliquot was subjected to ultrafiltration. An ultrafiltrate aliquot (200 μl) was loaded onto a previously conditioned SPE tube. The tube was then washed with 2 ml of a methanol/phosphate buffer (1 mM, pH 7) mixture (20:80, v/v) containing 1 mM TBABr and eluted with (200 × 3) μl aliquots of a methanol/phosphate buffer (1 mM, pH 7) mixture (80:20, v/v) containing 1 mM TBABr, discharging the first aliquot. The eluate was then evaporated to dryness and reconstituted with 40 μl of a methanol/water (50:50, v/v) mixture. Finally, 20 μl of the eluate were injected.

2.6. Determination of the total amount of MPA and MPAG

A 100 μl serum aliquot was added to 400 μl of 0.5 M HCl and 100 μl of the resulting mixture loaded onto a previously conditioned (see above) SPE tube. The tube was connected to a syringe filter containing a 3 mm diameter, 0.45 μm pore size nylon membrane (Supelco). The SPE tube was then washed with 2 ml of a methanol/phosphate buffer (5 mM, pH 7) mixture (20:80, v/v) containing 5 mM TBABr and eluted with 500 μl of a methanol/phosphate buffer (5 mM, pH 7) mixture (80:20, v/v) containing 5 mM TBABr. Finally, 20 μl of the eluate were injected.

3. Results and discussion

3.1. Chromatographic behaviour of MPA and MPAG

As a first approach, MPA and MPAG were tentatively separated as undissociated acids by controlling the pH of a methanol/aqueous buffer mobile phase. However, the chromatographic efficiency resulted always very poor, no matter the pH value and the methanol content used. Furthermore, MPAG was poorly retained and its capacity factor scarcely influenced by the eluent composition. Thus, the possibility of analytes separation as ion pairs, using a tetrabutylammonium (TBA) salt as ion pairing agent, was considered. The effect on retention of counterion concentration, nature of the organic modifier (MeOH versus ACN) and composition of the mobile phase, was studied first.

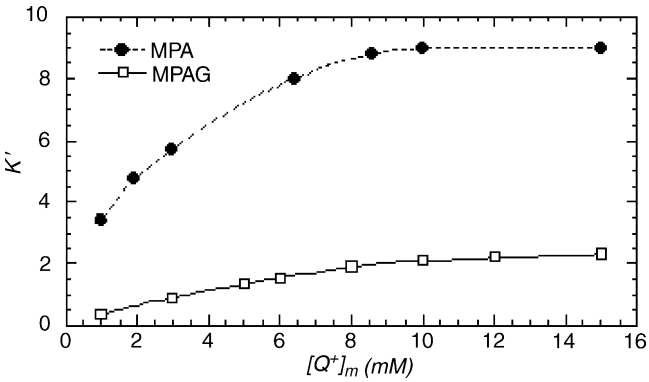

Fig. 1. Influence of the TBA concentration on MPA and MPAG retention at constant pH, ionic strength and volume fraction of organic modifier.

Fig. 1 shows the influence of the TBA concentration on MPA and MPAG retention at constant pH, ionic strength and volume fraction of organic modifier. As apparent, the retention can be regulated by the quaternary ammonium (Q^+) ion concentration up to ca. 10 mM. At higher TBA concentration, a plateau was observed, which can be due to a decreased capacity of the retaining phase to take up analyte (X^-) ion pairs (QX) caused by an increased adsorption of quaternary ammonium ion pairs.

The nature (MeOH or ACN) and the volume fraction of the organic modifier were then varied (at constant pH, ionic strength and TBA concentration) in order to evaluate the effects on retention and chromatographic efficiency for MPA and MPAG. Although both modifiers can be effectively used to regulate retention (the higher the volume fraction the lower the retention), methanol gave much better efficiency and peak shape than acetonitrile. A mobile phase composition of methanol/phosphate buffer (5 mM, pH 7) mixture (50:50, v/v) containing 5 mM TBABr gave theoretical plates number of 22.400 and 21.860 m^{-1} for MPA and MPAG, respectively and a peak asymmetry factors of ca. 1.18.

The retention behaviour of some organic anions as quaternary ammonium ion pairs on hydrophobic alkyl modified silica [22] or non polar poly(styrene-divinylbenzene) adsorbent [23] has been already described. When an analyte ion, X^-, is introduced in the chromatographic system above specified, it is distributed to the stationary phase as the ion pair QX, which competes with QC (where C^- is the co-anion of TBA^+) and QZ (where Z^- indicate a competing anion, e.g. $H_2PO_4^-$, in the buffer) for the available adsorption sites, A_s. Under certain

assumptions (negligible adsorption of X^- in the acidic form, capacity factor independent of X^- concentration) it can be shown [23,24] that retention can be expressed by Eq. (1):

$$\frac{1}{k'_X} = \frac{1}{qK_0K_{QC}K_{QZ}^{1/2}K_{QX}^{1/2}[Q^+]_m} + \frac{[C^-]_m}{qK_0K_{QZ}^{1/2}K_{QX}^{1/2}} + \frac{K_{QZ}[Z^-]_m}{qK_0K_{QZ}^{1/2}K_{QX}^{1/2}} \quad (1)$$

where k'_X is the capacity factor; q the ratio of solid phase to mobile phase in the column (g/1); K_{QC} the equilibrium constant for the process $Q_m^+ + C_m^- + A_s = QCA_s$; K_{QZ} the equilibrium constant of the process $QCA_s + Z_m^- = QZA_s + C_m^-$; K_{QX} the equilibrium constant for the process $QCA_s + QZA_s + 2X_m^- = 2QXA_s + C_m^- + Z_m^-$; $[Q^+]_m = TBA^+$ concentration in the mobile phase; K_0 is the monolayer capacity (i.e. maximum number of moles of ion pairs that can be taken up per gram of solid phase); m and s indicate mobile and stationary phases, respectively.

Eq. (1) shows that k'_X can be regulated by the mobile phase concentration of the counter ion (Q^+), of its co-anion C^- and of a competing ion of the same charge, Z^-.

Note that the third term in the right-hand-side of Eq. (1) indicates that $1/k'_X$ should increase on increasing $[Z^-]_m$ at constant $[Q^+]_m$ and $[C^-]_m$. However, no appreciable variation on the retention were observed by varying the phosphate buffer concentration between 1 and 10 mM. This indicates that under the experimental conditions used ($[Q^+]_m = [C^-]_m = 5$ mM; 1 mM < $[Z^-]_m$ < 10 mM) the buffer anion Z^- plays a very minor effect; this is not surprising considering that C^- (bromide in the present case) is the strongest eluent [22].

Note that at constant $[Z^-]_m$, an increase of TBABr concentration predict a decrease of $1/k'_X$, see first term in the right-hand-side of Eq. (1), and an increase of $1/k'_X$ due to the second term in the right-hand-side of Eq. (1). This means that k'_X should first increase, reach a maximum (see Fig. 1) and then decrease at very high QC concentrations (not observed in Fig. 1).

Obtaining $[C^-]_m$ from the equilibrium constant K_{QC}, and substituing in Eq. (1), the following equation can be readily obtained:

$$\frac{1}{k'_X} = \frac{1}{qK_0K_{QC}K_{QZ}^{1/2}K_{QX}^{1/2}[Q^+]_m}\left(\frac{QCA_s + A_s}{A_s}\right) + \frac{K_{QZ}[Z^-]_m}{qK_0K_{QZ}^{1/2}K_{QX}^{1/2}} \quad (2)$$

At low QC concentration in the mobile phase, QCA_s is much lower than A_s, the number of available sites for ion pair adsorption. Under this condition, the approximated form of Eq. (2) holds:

$$\frac{1}{k'_X} = \frac{1}{qK_0K_{QC}K_{QZ}^{1/2}K_{QX}^{1/2}[Q^+]_m} + \frac{K_{QZ}[Z^-]_m}{qK_0K_{QZ}^{1/2}K_{QX}^{1/2}} \quad (2')$$

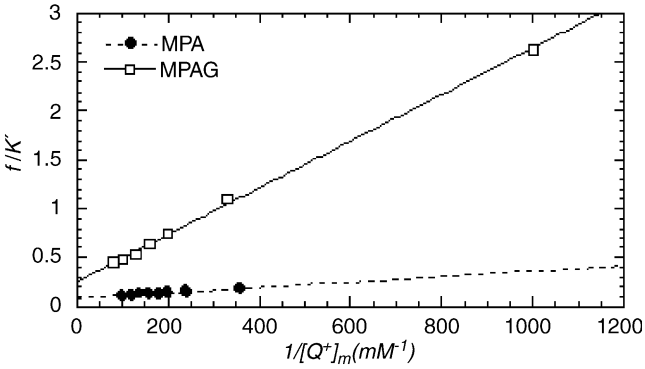

Fig. 2. Plot of $1/k'_X$ vs. $1/[Q^+]_m$ at constant phosphate (Z^-) concentration for MPA and MPAG.

Eq. (2′) shows that, at constant $[Z^-]_m$, a plot of $1/k'_X$ versus $1/[Q^+]_m$ should give a straight line as indeed verified in Fig. 2.

3.2. Analytical applications

The developed method was applied to the determination of MPA and MPAG in human serum samples. As already reported in Section 1, MPA and MPAG in serum are mostly bound to proteins (mainly albumin). Then, it was interesting to study the relationship between both total MPA and MPAG, between free MPA and MPAG, and between free and bound MPA concentrations.

3.2.1. Determination of free MPA and MPAG concentrations

Average recoveries, calculated by spiking drug free serum samples (n = 3) with MPA and MPAG at 0.5 and 10 μg/ml concentration levels, were (99.7 ± 0.2)% and (64.1 ± 6.9)%, respectively, and were not concentration dependent.

Calibration curves were constructed spiking serum samples with MPA and MPAG in order to cover the range from 0.06 to 1 μg/ml and 0.2 to 10 μg/ml, respectively; three replicates for each concentration were performed. Calibration curves resulted linear with correlation coefficients better than 0.995 and intercept not significantly different from zero at 95% confidence level. The within-day (n = 5) and between-days (n = 5 over 5 days) coefficients of variation, estimated by an ANOVA test, were 0.4 and 0.8% for MPA (at 0.1 μg/ml level) and 0.8 and 1.6% for MPAG (at 5 μg/ml level), respectively.

The LOD and LOQ in spiked serum, estimated according to IUPAC [25] recommendation, were 4 and 26 ng/ml for MPA and 17 and 60 ng/ml for MPAG, respectively. Note that LOD values for MPA compare well even with those obtained by tandem mass spectrometric detection or fluorescence detection after post column derivatization.

Fig. 3 shows, for instance, the chromatogram relevant to a serum sample taken from patient B after 1 h from MMF administration and treated as described in Section 2.

Although a pharmacokinetics study is outside the scope of the present paper, the obtained MPA and MPAG

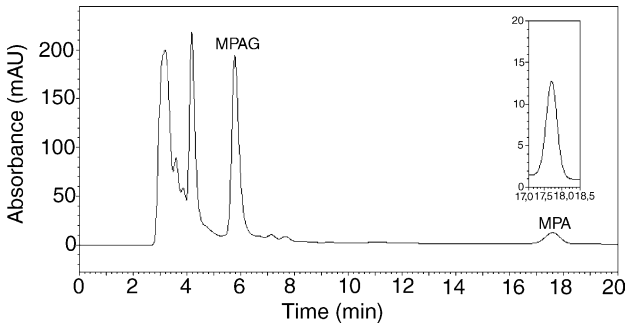

Fig. 3. HPLC–UV chromatogram relevant to the free MPA and MPAG determination in patient B after 1 h from MMF administration. MPA and MPAG estimated concentration: 0.09 and 3.9 μg/ml, respectively. Sample treatment and chromatographic conditions as specified in Section 2; detection at 254 nm. The inset shows MPA peak on a different absorbance scale.

concentration–time profiles in both patients under MMF therapy are shown in Fig. 4, just to demonstrate the potential of the described approach and the usefulness of free drug determination. It is interesting to note that although free MPAG concentrations in both patients are quite similar, the free MPA concentrations in patient B are about 10-fold higher than those of patient A. Since albuminemia status was similar in both patients, the difference in the MPA concentration could be ascribed to an alteration in the protein binding capabilities (altered binding sites structure) or to a different metabolism.

3.2.2. Determination of the total amount of MPA and MPAG

Average recoveries, calculated by spiking drug free serum samples (n = 3) with MPA and MPAG at 1 and 5 μg/ml concentration levels, were (99.3 ± 0.2)% and (50.1 ± 8.9)%, respectively, and were found concentration independent.

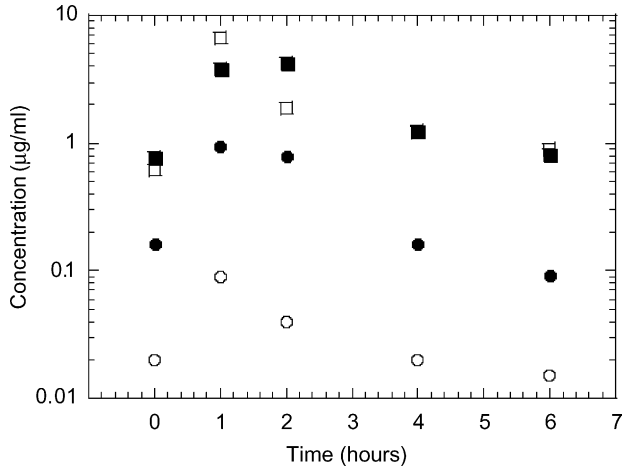

Fig. 4. Concentration–time profiles for free MPA and MPAG in both patient A and B under MMF treatment (1 g oral dose repeatedly administered at 12 h interval). Time starts immediately before a new dose administration. Symbols: (◯) MPA, patient A; (●) MPA, patient B; (□) MPAG, patient A; (■) MPAG, patient B. Note that the last point of the profile relevant to MPA in patient A is comprised between the LOD and the LOQ.

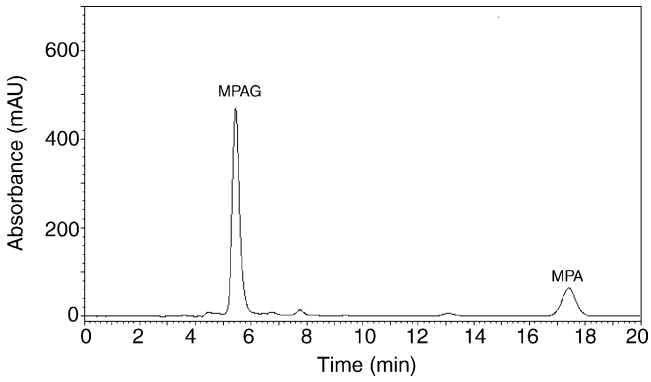

Fig. 5. HPLC–UV chromatogram relevant to the total MPA and MPAG determination in patient B after 1 h from MMF administration. MPA and MPAG estimated concentration: 8 and 78 μg/ml, respectively. Sample treatment and chromatographic conditions as specified in the experimental section; detection at 254 nm.

Calibration curves were constructed spiking serum samples with MPA and MPAG in order to cover the range from 0.2 to 10 μg/ml and 0.7 to 100 μg/ml, respectively; three replicates for each concentration were performed. Calibration curves resulted linear with correlation coefficients better than 0.996 and intercept not significantly different from zero at 95% confidence level. The within-day (n = 5) and between-days (n = 5 over 5 days) coefficients of variation, estimated by an ANOVA test, were 0.7 and 1.4% for MPA (at 5 μg/ml level) and 1.2 and 2.0% for MPAG (at 50 μg/ml level), respectively.

The estimated LOD and LOQ, calculated according to IUPAC [25] in spiked serum were 80 and 200 ng/ml for MPA and 350 and 650 ng/ml for MPAG, respectively.

Fig. 5 shows, for instance, the chromatogram relevant to a serum sample taken from patient B after 1 h from MMF administration and treated as described in Section 2. Fig. 6 reports the obtained MPA and MPAG concentration–time profiles in both patients under MMF therapy. As apparent,

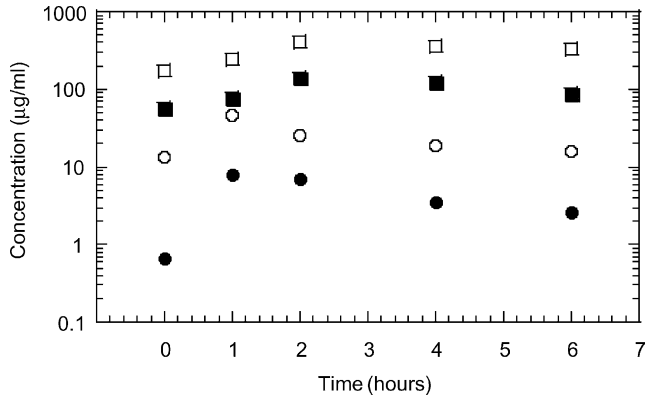

Fig. 6. Concentration–time profiles for total MPA and MPAG in both patient A and B under MMF treatment (1 g oral dose repeatedly administered at 12 h interval). Time starts immediately before a new dose administration. Symbols: (◯) MPA, patient A; (●) MPA, patient B; (□) MPAG, patient A; (■) MPAG, patient B.

both the total MPA and MPAG concentrations in patient B are higher then those found in patient A.

The free MPA fraction for both patients was then calculated as the ratio between the free and total concentration reported in the concentration–time profiles. The estimated average values were $(1.5 \pm 0.8)\%$ and $(0.7 \pm 0.2)\%$ for patient A and B, respectively. As apparent, the free MPA fraction was found similar in both patients, contrary to free and total MPA serum concentrations, that differ by one order of magnitude between the two patients. This experimental evidence further underlines the importance of an analytical method able to estimate free MPA concentration in order to optimize MMF dose to increase immunosuppression and minimize toxic effect.

4. Conclusions

The present HPLC–UV method is the first one permitting the simultaneous determination of free forms of MPA and MPAG in human serum samples.

A simple modification in the sample pretreatment procedure allows also the simultaneous determination of the total (free plus protein bound) concentration of MPA and MPAG. The potential of the method is demonstrated by excretion kinetics measurement in serum of patients receiving MMF therapy.

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