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Determination of the immunosuppressant mycophenolic acid in human serum by solid-phase microextraction coupled to liquid chromatography

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Abstract

A solid phase microextraction (SPME)–HPLC–UV method for the determination of the immunosuppressant mycophenolic acid (MPA) in human serum samples was developed for the first time. The procedure, that employed a carbowax/templated resin (Carbowax/TPR-100) as fiber coating, required a very simple sample pretreatment, an isocratic elution, and provides an highly selective extraction. The linear range was 0.2–100 $\mu g\,ml^{-1}$. Recovery was practically unchanged ($63 \pm 4\%$) passing from 0.2 to 100 $\mu g\,ml^{-1}$ level. Within-day and between-days coefficient of variation ranged from 5.9 to 6.5% and from 8.8 to 9.2%, respectively. A detection limit of 0.05 $\mu g\,ml^{-1}$ was estimated in spiked serum. The method was successfully applied to the determination of MPA in serum of a patient under mycophenolate mophetil ester (MMF) therapy, as demonstrated by the relevant concentration-time profiles.

Keyword: Mycophenolic acid

1. Introduction

Mycophenolic acid (MPA, see Fig. 1) represents a secondary metabolite produced by many species of moulds. First isolated from *Penicillium brevicompactum* and subsequently from other species (*P. roqueforti*, *P. viridicatum*, etc.), MPA is a reversible, non competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) and effectively blocks the de novo pathway of guanosine nucleotide synthesis, which leads to a reduction in DNA synthesis and proliferation of lymphocytes and subsequent immunosuppression [1]. Thus, MPA has been shown to be an efficient drug for the prevention of acute rejection in patients receiving organ transplantation [2–5].

A prodrug of MPA, the morpholinoethyl ester mycophenolate mophetil (MMF) has been synthesized to increase the bio-availability of mycophenolic acid. After oral administration, the ester is rapidly and completely adsorbed and hydrolyzed to its active metabolite (MPA) by plasma esterase. MPA is rapidly conjugated, mainly in the liver, by UDP-glucuronosyltransferase to its glucuronide (mycophenolic acid glucuronide, MPAG), that is pharmacologically inactive and primarily cleared through the kidney; however, it contributes to plasma concentration of MPA being hydrolyzed back to MPA by β-glucuronidase during enterohepatic recirculation.

Several analytical methods have been developed to determine MMF and/or MPA and/or MPAG in biological fluids, in particular plasma samples.

Existing papers [6–18] on this topic are mainly based on chromatographic separations following sample pretreatment by liquid–liquid (LLE) or solid-phase extraction (SPE) procedures that are, however, intrinsically laborious, time consuming and employ toxic solvents. These drawbacks can be avoided adopting solid phase microextraction (SPME), a new solventless technique introduced by Pawliszyn [19] for fast simultaneous extraction and pre-concentration of analytes from sample matrix.

SPME has been mainly applied [20–26] in combination with GC; however, SPME–GC is limited to the analysis of volatile and thermally stable compounds. In order to widen its range of application, SPME has been interfaced with HPLC [27]. A growing interest for SPME coupled to HPLC was observed, especially in the past few years,

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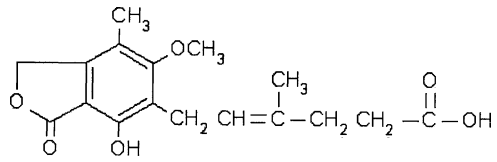

Fig. 1. Chemical structure of MPA.

as demonstrated by a number of recently published papers [28]. In particular, a new SPME–HPLC–UV method for the determination of the mycophenolic acid in cheese samples [29] was recently developed in our laboratory.

In the present paper, an SPME–HPLC–UV method for the determination of mycophenolic acid in human serum samples of patients undergoing pharmacologic treatment with MMF was developed for the first time. The potential of the method for carrying out pharmacokinetic studies was also demonstrated.

2. Experimental

2.1. Chemicals

MPA (6-(4-hydroxy-6methoxy-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 (1000 $\mu g\,ml^{-1}$) of mycophenolic acid 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. Working solutions for SPME were prepared in phosphate buffer (5 mM, pH 3) before use.

2.2. Apparatus

The SPME interface (Supelco, Bellefonte, PA), consisted of a standard six-port Rheodyne valve equipped with a fibre desorption chamber (total volume: 60 μl), installed in place of the sample loop. The HPLC system consisted of a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA), a Supelcosil LC–NH_2 column (250 mm × 4.6 mm i.d., 5 μm packing, Supelco), protected by a Supelguard LC–NH_2 precolumn (20 mm × 4.6 mm i.d., 5 μm packing, Supelco), and a photodiode-array detector (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer.

2.3. Chromatographic and detection conditions

The mobile phase used was an acetonitrile/ammonium acetate buffer (75 mM, pH 7) mixture (80:20, (v/v)) The flow rate was 1 $ml\,min^{-1}$ 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 234–358 nm range (5 nm band-width). Peak purity could be checked by the technique of spectra overlaying, after normalization. In the above described chromatographic system MPA and MPAG eluted at 7.5 and 5 min, respectively.

2.4. Solid-phase microextraction

A silica fiber coated with a 50 μm thick Carbowax/Templated Resin (CW/TPR-100, Supelco) was used. A manual SPME device (Supelco) was used to hold the fiber. The SPME device and procedure have been extensively described elsewhere [19,25] Working solutions were prepared by spiking 1.5 ml of phosphate buffer (5 mM, pH 3) with different amounts of MPA (or MPAG) into 2 ml clear vials (Supelco). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The extraction was carried out under magnetic stirring for 30 min at room temperature. Mycophenolic acid desorption into the SPME–LC interface was performed in the static desorption mode by soaking the fiber in mobile phase for 60 s. Then, the valve was changed to the inject position and the fiber was exposed for 4 s to the mobile phase stream.

In order to avoid possible memory effects the fiber was fully desorbed in "dynamic mode" before the next extraction.

2.5. Human serum samples

Drug free serum samples were collected from healthy volunteers; serum samples from patients under MMF therapy were obtained from a local hospital. 100 μl of each serum sample were added to 1400 μl of phosphate buffer (0.5 M, pH 3) and vortex mixed. The resulting mixture was filtered through a 5 μm Ministart sterile filter (Supelco) and finally subjected to SPME.

Recoveries were calculated as peak area ratio of MPA (buffer)/MPA (spiked serum samples). Serum samples were spiked with MPA at 0.2, 0.5, 1, 10 and100 $\mu g\,ml^{-1}$ concentration levels.

Quantitation was performed with the standard addition method. Calibration curves were constructed spiking drug free serum samples with variable amounts of MPA in order to obtain the following concentration levels: 0.2, 1, 10 and 100 $\mu g\,ml^{-1}$. Six replicates for each concentration were performed.

The within-day ($n = 5$) and between-days ($n = 5$ over 5 days) coefficient of variation for MPA were calculated on drug free serum samples spiked with variable amounts of MPA in order to obtain the following concentration levels at 0.1, 1, 10, 100 $\mu g\,ml^{-1}$ level.

3. Results and discussion

The effect of the most important parameters (e.g., extraction time, temperature, pH, ionic strength) influencing the

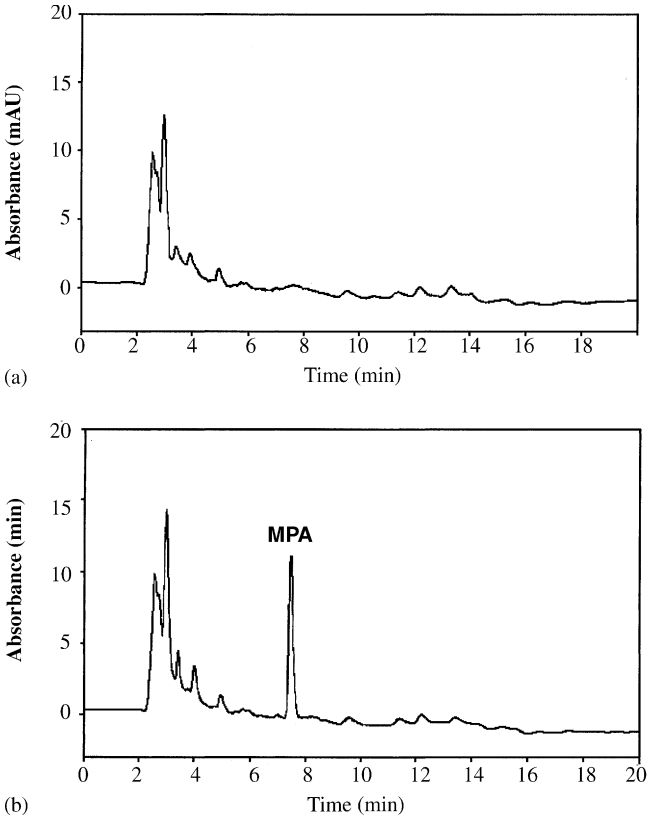

Fig. 2. SPME–HPLC–UV chromatograms of (a) a serum sample from a healthy donor and (b) the same sample spiked with MPA at a concentration level of 0.5 μg ml^{-1}. Chromatographic conditions and spectral acquisition as specified in the experimental section; detection at 254 nm.

extraction efficiency of MPA from phosphate buffer has been already discussed elsewhere [29] and can be summarized as follows. Ionic strength: significant (since an increase in the ionic strength of the solution was found to improve also the extraction of interfering compounds, no ionic strength adjustment of the phosphate buffer was performed); pH: significant (range explored from 3 to 11; best extraction efficiency at pH 3); extraction temperature: significant (range explored from 20 to 80 °C; best extraction efficiency at 20 °C); extraction time: significant (equilibrium reached at $t \geq 50$ min;). As for the choice of the fibre it was proved that the extraction efficiency of CW/TPR-100 coating was better than that of polydimethylsiloxane/divinylbenzene (PDMS/DVB). CW/TPR-100 fibres were then chosen for further investigations. MPAG was not extracted in none of the experimental conditions above described. Desorption conditions already employed for cheese samples [29], proved not adequate for serum samples and were then re-optimized.

Table 1
Within-day ($n = 5$) and day-to-day ($n = 5$, for 5 days) precision obtained on drug free serum samples spiked with variable amounts of MPA

MPA (μg ml^{-1})	Precision R.S.D. (%)	
	Within-day	Day-to-day
0.1	6.2	9.0
1	5.9	8.8
10	6.5	9.2
100	6.3	8.9

Fig. 2a and b reports the SPME–HPLC–UV chromatograms of (a) a serum sample from a healthy donor and (b) the same sample spiked with MPA at a concentration level of 0.5 μg ml^{-1}. As can be seen, in Fig. 2a, a very clean chromatogram was obtained despite of the very simple sample pre-treatment consisting in a 1:10 dilution of undeproteinized sample with pH 3 phosphate buffer. Note

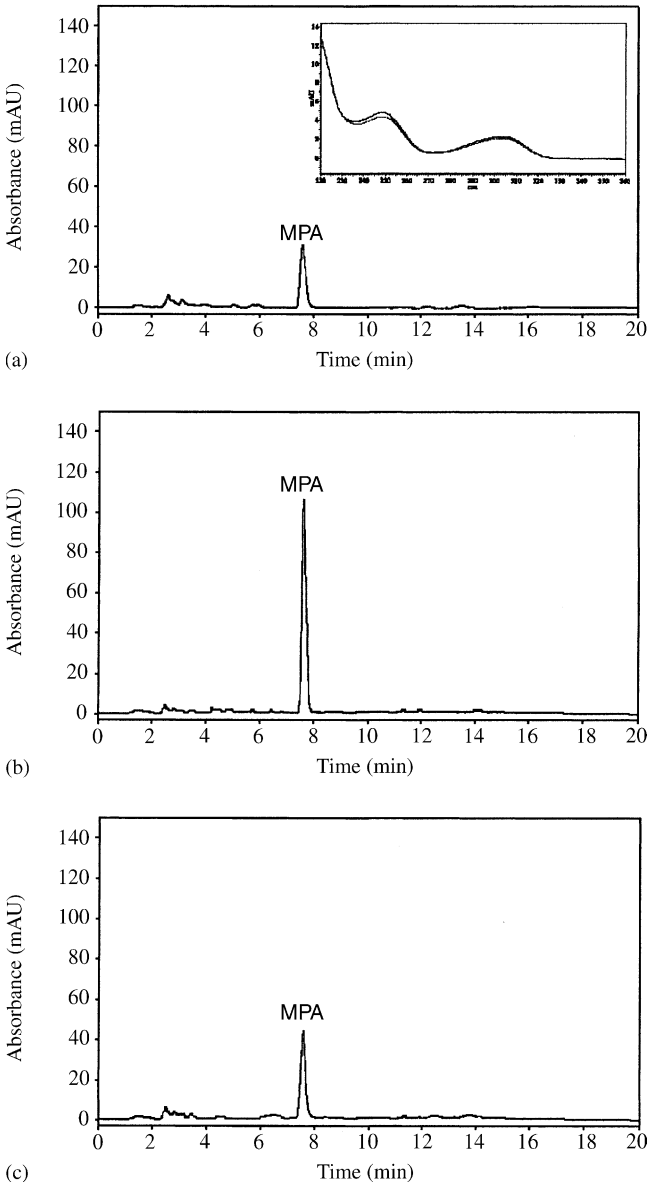

Fig. 3. SPME–HPLC–UV chromatograms of serum samples taken at different times: (a) immediately before a new dose administration (the inset reports the UV spectrum relevant to the peak of MPA); (b) and (c) 1 and 2 h after MMF administration, respectively, from a patient under MMF therapy. MPA estimated concentration: (a) 12.5 μg ml^{-1}; (b) 52.7 μg ml^{-1}; (c) 16.5 μg ml^{-1}. Chromatographic conditions and spectral acquisition as specified in the experimental section; detection at 254 nm.

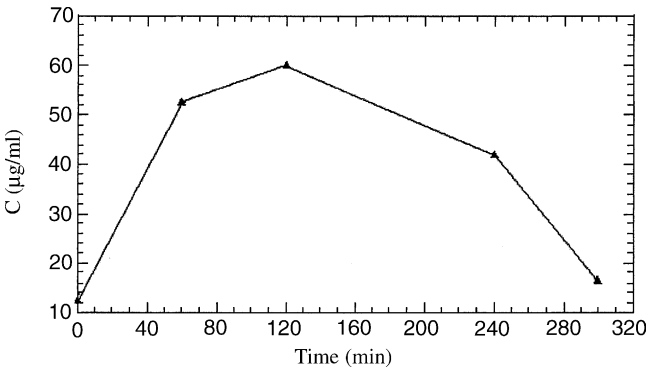

Fig. 4. Concentration–time profiles for MPA in a patient under MMF treatment (repeatedly administered oral dose (1 g) at 12 h interval). Time start immediately before dose administration.

that MPA in serum is mostly bound to proteins [30] (mainly albumin). Since the fiber can extract only free MPA, the sample treatment must ensure the shift of the equilibrium (which is pH dependent) towards the free form of the drug. As can be inferred by the following recovery data, the employed acidic buffer ensures a satisfactory release of protein bound MPA; the measured amount should then be referred to the total (free plus protein bound) form.

The obtained recovery was $63 \pm 4\%$ ($n = 5$) and remained practically unchanged passing from 0.2 to 100 $\mu g\,ml^{-1}$ level. MPAG added to serum samples at levels ranging from 2 to 200 $\mu g\,ml^{-1}$ could not be recovered to any extent since it was unextracted in the SPME step.

Calibration curve resulted linear in the range 0.2–100 $\mu g\,ml^{-1}$ with correlation coefficients better than 0.998 and intercept not significantly different from zero at 95% confidence level.

Table 1 reports the obtained within-day and between-days coefficient of variation for MPA.

The estimated LOD and LOQ in spiked serum obtained in this study were 0.05 and 0.12 $\mu g\,ml^{-1}$, and were calculated according to IUPAC [31].

Such limit of detection seems adequate for the monitoring of MPA in serum of patients under MMF therapy [32] with repeatedly administered dose at 12 h interval.

To demonstrate the usefulness of the proposed method for "real" samples analysis, serum specimens taken at different times from a patient under MMF therapy have been processed and analyzed. Fig. 3a–c shows the typical SPME–HPLC–UV chromatograms (the inset of Fig. 3a reports the UV spectrum taken on the apex of MPA peak). As apparent, MPA deriving from MMF metabolism can be easily detected and quantitated.

Although a pharmacokinetic study is outside the scope of the present paper, an example of the obtainable concentration-time profiles of MPA in a patient under MMF therapy is shown in Fig. 4, just to demonstrate the potential and soundness of the described approach. The time profile has the typical shape expected for the particular administration scheme of MPA [32].

4. Conclusions

An SPME–HPLC–UV method for the determination of the immunosuppressant MPA in human serum samples was developed for the first time. The procedure requires the simplest sample pretreatment developed up to now on this topic and it has also a comparable sensitivity with respect to existing methods devised to determine the total amount of MPA in serum of patients undergoing MMF therapy.

The free drug (the pharmacologically active form) is typically a few percent of the total MPA but could show extreme inter-patient variability. This means that the direct measurement of the free form could be even more significant; work in this direction is in progress in our laboratory.

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