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Development and validation of an LC/MS/MS assay for mycophenolic acid in human peripheral blood mononuclear cells

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Abstract

The aim was to develop a LC/MS/MS method able to quantify mycophenolic acid (MPA) in the peripheral blood mononuclear cells (PBMCs) of transplanted patients. PBMCs were isolated from blood by a density gradient separation. The chromatographic separation was carried out on a Zorbax Stable Bond CN, 150 mm × 2.1 mm, and MS/MS detection was performed after positive electrospray ionisation of the protonated parent ion. The calibration range was from 0.25 to 100 ng/sample. Extraction from the cells and ionisation recoveries reached 73.5 and 37.9%, respectively. Inaccuracy was always <10% with CVs <15%. MPA was stable at room temperature in the autosampler over 48 h and at −20 °C over 1.5 months. Application to clinical samples taken from patients treated with mycophenolate mofetil indicated that the method is suitable for measuring intracellular MPA.

Keywords: Mycophenolic acid; PBMC; Intracellular; Tandem mass spectrometry; Clinical pharmacology; Patient

1. Introduction

Mycophenolate mofetil (MMF) is an immunosuppressant widely used in current standard immunosuppressive therapies in renal, liver, pancreas and heart transplantation in combination with calcineurin inhibitor (CNI) and have been demonstrated to reduce acute rejection (AR). The drug appears as a cornerstone of most of the current regimen therapy to prevent AR. After reaching the bloodstream, MMF is hydrolysed by blood esterases to mycophenolic acid (MPA), the active drug. Attempts to correlate MPA concentrations with toxic events, such as severe diarrhea or neutropenia have been made by measuring plasma MPA levels in clinical samples. Large intra- and inter-patient variability in systemic MPA pharmacokinetics [1] have prompted attempts at therapeutic drug monitoring. However, the link between plasma MPA levels and drug response is not easy to establish [2]. Some authors have however succeeded to correlate total AUC [3], truncated AUC [4] or population pharmacokinetics and Bayesian estimation of mycophenolic acid [5] with patient outcome. Such sampling schedule or data management remain quite involved. Since most MPA (>90%) is bound to albumin, variation of albumin concentration may affect the free fraction of MPA leading some pharmacologists to measure the free drug which is the pharmacologically active form [6]. However, the ideal strategy for therapeutic monitoring of MPA has yet to be fully defined and does not address the level of drug in the target cells. Thus, a new tool for TDM to better predict the clinical event is still needed.

In its target cells (T and B lymphocytes), MPA inhibits intracellular inosine monophosphate dehydrogenase (IMPDH), leading to a decrease in endogenous nucleotides, especially purine nucleotides, necessary for DNA synthesis. Thus, the clinical pharmacology of drugs that act on circulating blood cells may require description of drug behaviour inside the cells. For such drugs (antiretrovirals, anticancer agents, immunosuppressants), intracellular pharmacology and metabolism are considered to be a relevant approach to understand the lack of efficacy and/or toxic events. For this purpose, sensitive and specific assays are

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needed. Recently, several assays suitable for the detection and quantification of antiretroviral or anticancer drugs in human PBMCs [7–9] have been described, and recommendations have been made for the handling of cells and assay validation [10,11].

Several methods have been described for the quantification of MPA in plasma, including liquid chromatography with UV detection [12–14] and EMIT. Due to quite high plasma MPA levels (i.e., μg/mL), sensitivity and chromatographic efficiency were not particularly stringent. Conversely, since intracellular drug concentrations may be very low, especially if the sample contains few cells, assay sensitivity is of major concern during the development phase. Liquid chromatography tandem mass spectrometry (LC/MS/MS) has been used successfully to quantify free MPA and total MPA [15] or its glucuronide metabolite in plasma [16–18], with better accuracy than immunoassay [19] and should be applied to intracellular drug assay.

Here, we describe an assay suitable for the quantification of intracellular MPA in human PBMCs, and after validation, for use in patients on MMF therapy.

2. Experimental

2.1. Chemicals

Mycophenolic acid or MPA ($C_{17}H_{20}O_6$/MM: 320.34) and the ISTD ketoprophen ($C_{16}H_{14}O_3$/MM: 254.03) were from Sigma (Sigma Chemical Co., St Louis, MO, USA) (Fig. 1).

2.2. Materials and reagents

Human blank blood from healthy subjects was obtained on citrate, phosphate, dextrose (CPD) as anticoagulant from EFS (Rungis, France). A CR312 centrifuge (Jouan, St. Herblain, France) was used to isolate plasma from blood and PBMCs were separated using cell preparation tubes (CPTs) from Becton Dickinson (Le Pont de Claix, France). HPLC quality acetonitrile was from SDS (Peypin, France), methanol from VWR (France) ultrapure water from a Maxima II purifier (USF ELGA, France). Nitrogen U was from Messer (France). Eppendorf 1.5 mL safe lock cones were used to keep samples. 20 mmol/L ammonium formate pH 2.5:1.26 g of ammonium formate was dissolved in 1 L of ultrapure water, the pH was adjusted to 2.5 with formic acid. The 1 M buffer Tris–HCl buffer pH 7.4 lysis solution was made as follows: 12.11 g of Trizma base was dissolved in 85 mL of ultrapure, the pH was adjusted to 7.4 with HCl, and the solution was diluted to 100 mL with ultrapure water.

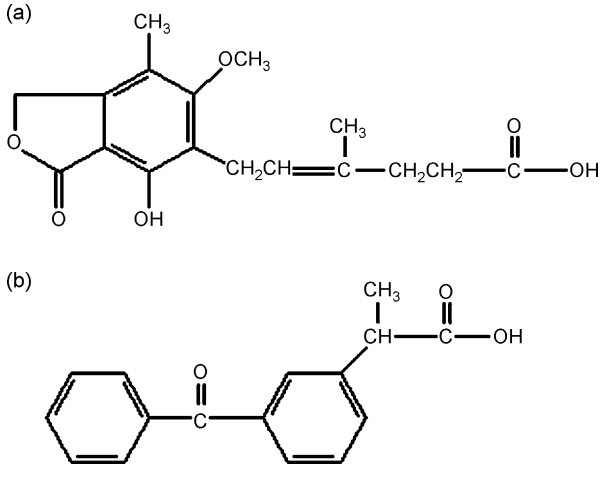

Fig. 1. Structures of MPA (a) and ketoprophen (b).

2.3. Instrumentation

Chromatography was done with an HP1100 (Agilent Technologies) connected to a Quattro Micro tandem mass spectrometer equipped with an electrospray source (Waters/Micromass, St. Quentin en Yvelines, France). This instrumentation was monitored using Masslynx 4.0 acquisition and data treatment software (Waters/Micromass).

2.4. Stock and diluted standard solutions

Stock standard solutions of MPA were prepared at 1 mg/mL in methanol. After dilution to 10 μg/mL in methanol, diluted solutions were prepared at target concentrations for calibration standards and quality controls. Internal standard solution at 10 μg/mL then diluted to 2500 ng/mL was prepared in methanol. The standard solutions were stored at +4 °C for 1 month without degradation.

2.5. Preparation of calibration standards and quality controls, and extraction procedure

Calibration standards and quality controls covering the concentration range from 0.25, 0.5, 25, 50, 75, 100, and 0.6, 40, 80 ng/sample, respectively, were prepared by adding 20 μL of diluted solution of standard, 20 μL of internal standard diluted solution (50 ng) to a blank PBMC pellet containing approximately 10×10^6 cells.

PBMCs were lysed after addition of 1 mL of Tris–HCl buffer previously stored at −20 °C, as lysis solution, by several manual scraping of the tubes containing the cells against a metallic tray in order to produce cell membrane damaging. The process has shown to lyse completely the cells as checked by a visual examination with a microscope. After centrifugation (18,000 × *g* at 4 °C), the supernatant was transferred and evaporated to dryness in approximately 30 min. The dry residues were reconstituted in 50 μL of 20 mmol/L ammonium formate pH 3.6/acetonitrile 85/15 (v/v) and after vortex mixing, 20 μL were injected into the chromatographic system.

2.6. LC/MS/MS conditions

2.6.1. HPLC conditions

Chromatography was done on a Zorbax Stable Bond CN, 150 mm × 2.1 mm, 5 μm particle size thermostated at 40 °C and protected by a filter (2 μm) (Agilent Technologies, France). The mobile phase was 20 mmol/L ammonium formate pH 2.5/acetonitrile 72/28 (v/v) delivered at 400 μL/min. The retention times were 5.7 and 6.1 min for MPA and internal standard,

respectively, and the total run time was 10 min. From 0 to 4 min and from 8 to 10 min and using a divert valve, the mobile phase was diverted outside the MS source while an additional LC pump delivered water/acetonitrile/formic acid 75/25/0.1 at 400 μL/min. This washing step lowered the amount of endogenous compounds eluted in the solvent front and at the end of the analysis able to make the source dirty.

2.6.2. MS/MS conditions

After chromatographic separation, the mobile phase was directly introduced in the mass spectrometer via an electrospray source (ESI) operating in the positive mode. Nitrogen was employed as desolvation and nebulisation gases and argon was employed as collision gas. The acquisition mode used was multiple reaction monitoring (MRM). To observe how the potential settings affect primary and fragment ions, analytes were directly infused into the instrument using a syringe pump, at the concentration 1 μg/mL prepared in 20 mmol/L ammonium formate pH 2.5/acetonitrile 72/28 (v/v). The *m/z* 320.7 and 254.9 were the most sensitive ions in the Q1 mass spectra of MPA and ISTD, respectively. After fragmentation, the MS/MS product ion spectra indicated that the more intense product fragments were *m/z* 206.7 and 104.8 for MPA and ISTD, respectively. The dwell time was 0.5 s. Tuning parameters were 30 V and 20 eV as cone voltage and collision energy, respectively.

2.6.3. Validation conditions

The method validation followed the recommendations for bioanalytical method validation [20] and the parameters investigated were similar to those reported for a similar intracellular assay [10].

2.7. Calibration curve

The calibration curve was constructed with six levels ranging from 0.25 to 80 ng/sample. The first and last calibration standards (lowest and highest) were injected in duplicate either at the beginning or the end of the run with two other calibration standards (i.e., levels 1, 3, 5 and 6 at the beginning and levels 1, 2, 4 and 6 at the end of the run). The standard curve fitted linearly $Y = aX + b$ with $1/X$ as weighting factor, where X was the amount of MPA and Y the ratio of the chromatographic peak areas (MPA/ISTD).

2.8. Accuracy and precision and limit of quantification

Intra- and inter-day precision and inaccuracy were evaluated using 0.25 (LOQ), 0.6, 40 and 80 ng/sample. Five replicates of QC samples from each of the four concentrations were assayed in one run for the intra-day experiments. One QC sample from each of the four concentrations was assayed on 6 different days (corresponding to six runs) for the inter-day experiments. Means, standard deviations and coefficients of variation (CV%) were calculated. Precision was evaluated as the relative standard deviation of the mean expressed as a percent (coefficient of variation: CV%). Inaccuracy was expressed as the absolute percent deviation from the theoretically determined concentration (% difference). Limit of quantification (LOQ) was assumed to be the smallest amount of analyte likely to be quantified accurately (<20% deviation) and precisely (<20% CV).

2.9. Recovery

Total recovery represents two components: PBMC lysis recovery and ionisation recovery. Recoveries were assessed by comparing the MPA/ISTD peak area ratio measured after spikes ($n = 5$) at 0.65, 43.4 and 86.7 ng/sample on blank PBMC before, after lysis and in the injection solvent. The lysis recovery was determined by comparing peak area ratios in PBMC samples spiked before and after lysis. The ionisation recovery (i.e., also called matrix effect) was determined by the post-lysis (extraction) addition method, i.e., comparing the peak areas in PBMC sample spiked after lysis and in mobile phase.

2.10. Influence of matrix effect on accuracy and precision at the LOQ

The influence of the matrix effect on the accuracy was studied at the LOQ (exactly 0.24 ng/sample) by spiking six PBMC pellets of different origins with the same amount of MPA. The CV% of precision and the accuracy were calculated.

2.11. Stability

The stability of MPA spiked in blank processed samples was established at two amounts (0.65 and 86.7 ng/sample) after storage at −20 °C for 1.5 and 6 months by the comparison with freshly spiked processed samples. Moreover, the stability of MPA in the autosampler kept at room temperature for 24, 48 and 72 h was also studied at two different levels (0.65 and 83.7 ng/sample). The stabilities were expressed as the % of starting concentration. For the two experiments, three replicates were analysed at each concentration level.

2.12. Application to patients

The bioanalytical method developed here was applied to cellular clinical samples collected from patients treated with MMF as part of their immunosuppressive therapy. After blood collection (around 7 mL) in Vacutainer® CPT™ tubes, the PBMCs were separated by centrifugation through the density gradient gel according to the manufacturer recommendations and the PBMCs layer was carefully collected. PBMCs were washed twice in cold (4 °C) 0.9% NaCl, centrifuged at 1800 × *g* over 5 min. PBMCs were lysed (Tris–HCl buffer and manual scraping) and MPA was extracted from the cells and analysed according to the procedure described in Section 2.5. PBMCs were counted according to a previously validated biochemical method [21]. Briefly this method is based on the linear relationship between the PBMCs count and a fluorescence signal generated during detection of DNA in the cell pellet. The procedure involves digestion in 1 N NaOH, and fluorescence detection after addition of SYBr green in microplate. The calibration curve constructed between the cellular counting (ranging between 1.56 and 25 × 10^6 PBMCs) and

the fluorescence allows the cell count determination in clinical samples. The method has been validated in terms of precision, accuracy and stability. Thanks to the cell count, the MPA concentrations were calculated in ng/10^6 PBMC. The patients participated after the approval of the Ethics Committee (Bicêtre Hospital, France).

3. Results

3.1. LC/MS/MS chromatographic characteristics in spiked and clinical samples

The retention times of MPA and internal standard (ISTD) were around 5.7 and 6.1 min, respectively.

MRM chromatograms of MPA in an intracellular extract blank, spiked at the LOQ (0.24 ng/sample), and of a clinical sample (19.6 ng/sample or 2.5 ng/10^6 cells, since the cell count is 7.86×10^6 cells) are shown in Fig. 2. No endogenous substances interfered with any of the analytes in the blank sample and the LOQ was well detected from the baseline. The same informations are given for the ISTD in Fig. 3.

3.2. Calibration curves

There are several ways to plot a calibration curve for an intracellular assay. Clinical sample concentrations cannot be read directly from a calibration curve in ng/10^6 cells, since the cell count differs in each sample. The solution chosen by us and others [22] was to use a ng/sample-calibration curve, each standard being prepared from around 10×10^6 cells and spiked with the desired amount of MPA.

For clinical samples, the calibration curve allowed to obtain the MPA amount (in ng/sample) in each sample containing various numbers of cells. Then, and using the cell count (in 10^6 cells), a single division was performed in order to express the MPA concentration in ng/10^6 cells.

The data were well fitted with the linear curve as indicated by *R* values always higher than 0.99 (see Table 1).

3.3. Precision, accuracy and limits of quantification

Intra- and inter-assay precision and accuracy with a lower limit of quantification (LOQ) for MPA set at 0.24 ng/sample (corresponding to 100 pg injected), are reported in Table 2. Intra- and inter-assay CV% for precision was always lower than

Table 1
Standard curve parameters

	Slope	Intercept	Correlation coefficient
	0.0368593	−0.00155050	0.995843
	0.03515	−0.00145500	0.9991
	0.0328574	0.00104450	0.996015
Mean	0.0349556	−0.0006537	0.9969860
CV [%]	5.7	[a]	0.2

[a] CV of coefficient of correlation not applicable due to non-normal distribution of this measure.

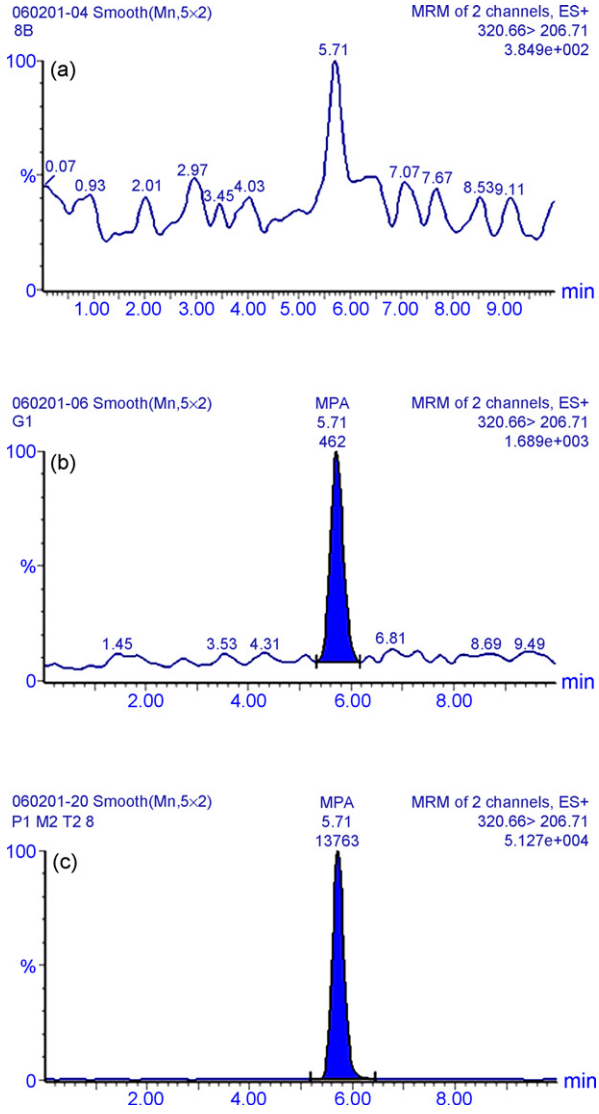

Fig. 2. Typical MRM chromatograms of (a) MPA signal in a blank PBMC extract, (b) MPA standard at LOQ (0.25 ng/sample) and (c) a clinical sample taken from a patient on MMF therapy (concentration: 19.6 ng/sample or 2.5 ng/10^6 cells).

Table 2
Precision and accuracy of the assay

	Nominal concentration (ng/sample)			
	0.238	0.65	43.4	86.7
Intra-assay precision				
Mean calculated amount (ng/sample)	0.268	0.628	42.9	80.7
SD	0.022	0.053	2.12	5.74
CV%	8.0	8.4	5.0	7.1
Intra-assay accuracy (%)	113	97	99	93
Inter-assay precision				
Mean calculated amount (ng/sample)	0.275	0.684	40.7	85
SD	0.006	0.06	1.47	7.62
CV%	2.1	8.8	3.6	9
Inter-assay accuracy (%)	115.5	105.3	93.8	98

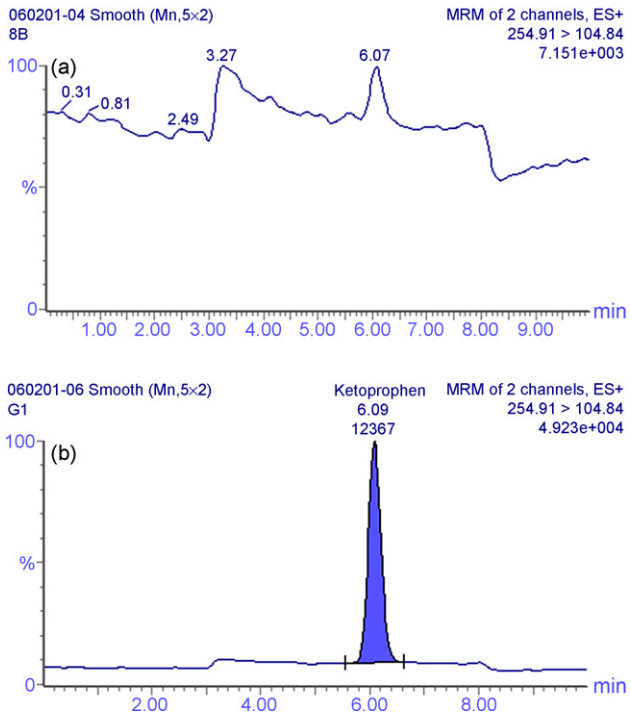

Fig. 3. Typical MRM chromatograms of (a) ketoprophen (ISTD) in a blank PBMC extract and (b) a clinical sample (spiked at 50 ng/sample).

10% and accuracy was between 93.1 and 112.7% and between 93.8 and 115.6% for intra- and inter-assay, respectively. Limit of quantification was set at 0.24 ng/sample (corresponding to 100 pg injected) with CV% of 8.0 and 2.1% for intra- and inter-assay precision, respectively, and intra- and inter-assay inaccuracy of 12.7 and 15.6%, respectively. Surprisingly, the inter-assay (CV 2.1%) was better than the intra-assay precision (CV 8.0%) at the LOQ. This is due to the variability of intra-assay precision itself since it was measured at one occasion.

3.4. Recovery

Cell lysis, ionisation and total recoveries are shown in Table 3.

For MPA (ISTD, ketoprophen), mean cell lysis and ionisation recovery reached 78% (92.3%) and 36.1% (40.7%), respectively, with no differences as a function of the amount of MPA. Thus, total recovery was 28.1 and 37.6% for MPA and ISTD, respectively.

Table 3
Recovery (lysis or extraction and ionisation) of the assay ($n = 3$ for each value)

	Nominal amount (ng/sample)			ISTD
	0.65	43.4	86.7	
Lysis recovery (%)	73.5	87.6	73.0	
Mean lysis recovery (%)		78		92.3
Ionisation recovery (%)	37.9	35.9	34.4	
Mean ionisation recovery (%)		36.1		40.7
Total recovery (%)	27.8	31.4	25.1	
Mean total recovery (%)	28.1			37.6

Table 4
Precision and accuracy at the LLOQ

Theoretical amount (ng)	0.238	
	Calculated amount (ng)	Accuracy (%)
Subject 1	0.303	127.3
Subject 2	0.277	116.4
Subject 3	0.292	122.7
Subject 4	0.271	113.9
Subject 5	0.296	124.4
Subject 6	0.260	109.2
Mean	0.283	119.0
Standard deviation	0.016	6.927
CV (%)	5.8	

3.5. Matrix effect at the LOQ

The matrix effect had no effect at the LOQ (0.24 ng/sample) since for matrix of six different origins the mean amount of MPA was 0.28 ng/sample, with a CV% for precision of 5.8% and a mean accuracy of 119% with five/six samples within 25% (see Table 4).

3.6. Stability of MPA processed samples stored at −20 °C and at room temperature on the autosampler

The stability of MPA in processed sampled extracts stored frozen at −20 °C is shown in Table 5. The mean accuracy was higher than 90% over 2 months. The stability of MPA on the autosampler over 72 h at room temperature is shown in Table 5. The mean accuracy was higher than 90% over 48 h, but decreased between 48 and 72 h.

In an intracellular assay, the *stricto sensu* stability study of the drug using spiked sample is not relevant since the drug is located inside the cells. That is why we have spiked processed samples extracts (i.e., after cell lysis). Nevertheless, the stability of MPA in biological fluids has been well established in the literature and MPA is stable at −20 °C in whole blood and in plasma for

Table 5
Stability under various storage conditions ($n = 3$ for each value)

Storage conditions		Theoretical amount (ng/sample)	Percentage of initial
Autosampler room temperature	24 h	0.65	90.2
		86.7	87.9
Autosampler room temperature	48 h	0.65	89.1
		86.7	93.1
Autosampler room temperature	72 h	0.65	81.4
		86.7	79.8
Cell extract at −20 °C	1.5 months	0.65	105.8
		86.7	108
Cell extract at −20 °C	6 months	0.65	51.2
		86.7	66.5

at least 11 months and in urine for at least 6 months and also after three freeze-thaw cycles [23] and it is worth nothing that MPA should be stable inside the cells stored at −20 °C over at least 2 months.

4. Discussion and conclusion

The aim of this work was to develop and validate an assay for intracellular mycophenolic acid (MPA) in human PBMCs which would be suitable for clinical pharmacology purposes. The relationship between plasma levels and drug response or toxic events is not easily demonstrated and routinely relevant. Thus, with the aim of searching for more relevant parameters, we decided to monitor MPA at its site of action, i.e., in lymphocytes. PBMCs including mostly lymphocytes and few monocytes were chosen as the biological medium. PBMCs can be easily separated from the other blood constituents (red blood and polynuclear cells) after blood collection in appropriate collection tubes (Vacutainer® CPT™) by a single centrifugation step in order to be used in current clinical situation and routinely. After two washing steps in 0.9% NaCl, PBMCs are frozen and sent to the analytical laboratory for assay of intracellular MPA and PBMCs counting. The PBMCs counting is therefore carried out at the end of the analytical process once the cells have been lysed and on the cell pellet DNA-containing.

Among the available analytical methods, LC/MS/MS appeared to be the method of choice even though it is still not widespread in hospital pharmacological laboratories. Immunoassays revealed a systematic positive bias with regard to HPLC due to cross-reactivity with glucuronide metabolite [24]. For clinical pharmacology purposes, time can be saved by avoiding the widely used extraction step (liquid/liquid or solid phase extraction) especially when the drug concentration is high. Fortunately, cell lysis using the Tris–methanol buffer precipitates intracellular proteins and the extract can therefore be injected into the LC system without additional extraction step. To counterbalance the lack of extraction step with respect to ionisation recovery, the LC was developed in order to separate MPA from a panel of endogenous compounds eluted in and near the solvent front. Thus, the retention time of MPA and ISTD (ketoprophen) was 5.7 and 6.1 min, respectively, which represents a k' of around 3.

Using liquid chromatography tandem mass spectrometer, the assay was specific with respect to endogenous compounds and associated drug therapies. Analysis of samples from several patients treated with MMF as part as their immunosuppressive therapy did not reveal any other chromatographic peak able to interfere with the assay (data not shown).

Since the matrix effect, measured by the ionisation recovery, is a common problem in LC/MS/MS, it was calculated and its influence on assay precision and accuracy was investigated. Despite a quite low total recovery (mean: 28.1%) mainly due to the ionisation recovery (mean: 36.1%), the matrix effect had no impact on the precision and accuracy of the assay thanks probably to the ISTD chosen. Although not a stable labelled isotope, ketoprophen is also a carboxylic acid and has a retention time close to that of MPA. Attention should be paid to patients with rheumatoid arthritis since ketoprophen can be used in these patients.

In order to check that the method is robust despite a low ionisation recovery, we have measured the signal of the ISTD in three samples of the first 10 patients analysed with the assay. The CV% of the chromatographic MRM peak area was 9.3 and 19% for intra- and inter-subject, respectively, indicating that the variation of the signal is acceptable. Moreover, the retention times of MPA and ISTD are close (5.7 and 6.1 min, respectively) giving relevancy to this information. Nevertheless, the assay is potentially vulnerable to matrix effect and the ISTD signal has to be monitored carefully.

Regarding the ionisation, both MPA and ISTD are surprisingly better ionised in the positive mode and the protonated ions are fragmented leading to stable fragment at *m/z* 207 and 105, respectively. Thus, when low amounts of MPA were spiked in cell extracts of six healthy subjects, the inaccuracy % deviation and the precision (CV%) were very low. Despite this quite low total recovery, the assay sensitivity is sufficient adequate to quantitate MPA in patients' PBMCs with a large ability (see Fig. 2).

For the chromatographic step system that was not underestimated as too often meet, several chromatographic columns with octadecylsilane (C18) or cyano (CN) as bonded groups were tested. Best results were obtained with a Zorbax Stable Bond CN. Due to endogenous compounds eluted in the solvent front, it was desirable to obtain a k' at around 3, corresponding to retention times of around 6 min, to separated MPA from these endogenous compounds. The run time is 10 min and more than 100 clinical samples can be analysed in each analytical run.

In the analytical method presented in this paper, a high sensitivity was sought by testing several combinations of mobile phase/ionisation mode. The first idea was to use the negative mode to ionise the MPA (which possesses a carboxylic group) coupled with a neutral mobile phase. This classical approach has been chosen by others in either electrospray (ESI) [18] or atmospheric pressure chemical ionisation (APcI) [25] as ionisation source, the authors using an API 2000 and API III, respectively. However, in our hands and with the MS system used (Quattro Micro, Waters/Micromass), a more efficient system of mobile phase/ionisation source and mode was achieved with ammonium acetate in the mobile phase and electrospray ionisation in the positive mode. Several previous papers describe the use of ammonium adduct using a Quattro Micro [16] an API [15], and an API III [17] but in our hand and using a Quattro Micro we have found that the protonated adduct ion was the more sensitive. The limit of quantitation was lowered to 0.24 ng/sample, which corresponds to around 100 pg injected into the LC/MS/MS system, which is largely sufficient to monitor intracellular MPA as seen in the first analysis. Note that two injections are possible since 20 out of 50 μL of the solution are injected. Around 60 scans were used for chromatographic peak definition, since the peak eluted within approximately 1 min and the cycle time was 1 s. This cycle time is definitely relevant for an accurate peak area determination.

All the usual validation tests of precision and accuracy, and linearity in the calibration range easily met the recommended

criteria established previously [20], and assay reliability was not affected by electrospray-related ionisation recovery (i.e., matrix effect) and the mass spectrometric performance of the assay was quite reliable. Regarding specificity, it had been noticed previously that the glucuronide metabolite of MPA (MPAG) was able to interfere with the MPA determination since an in-source fragmentation of the glucuronide occurs [26]. We have checked with authentic MPAG (ROCHE diagnostics GmbH, Mannheim) that MPAG did not interfere with the detection and quantification of MPA in this assay.

It is worth nothing that once thawed and lysed, cells cannot be refrozen and the classical freeze/thaw cycle experiment is therefore not relevant as previously discussed [10]. MPA was stable in cell extracts reconstituted in the injection solvent stored at −20 °C over 1.5 months, but not over 6 months. Regarding stability in cells, this step is really difficult to clearly be demonstrated since spike samples do not described accurately the presence of the drug inside the cells. Several paper have previously reported the stability of MPA as well as MPAG after several freeze/thaw cycles [18,23].

Thus, in this paper, we present an LC/MS/MS assay suitable for the intracellular quantification of the widely used immunosuppressive drug MPA, with the required confidence levels. This assay can be applied to clinical pharmacology or research in any analytical laboratory possessing a tandem mass spectrometer. Further studies are under way to evaluate the reliability of intracellular measurement of MPA in the clinical setting.

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