Analytical and Pharmacological Aspects of Therapeutic Drug Monitoring of mTOR Inhibitors

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Abstract: Mammalian Target Of Rapamycin (mTOR) inhibitors represent a new class of immunosuppressant drugs extensively used for the prevention and the treatment of graft rejection in organ transplant recipients. Their current use is due to referred low nephrotoxic effects, particularly important in kidney transplanted and/or patients with renal failure. The most representative drugs of such class are Sirolimus (Siro) and Everolimus (Rad). Both drugs show a narrow therapeutic window, therefore, monitoring of whole-blood drug levels is recommended in order to optimize the therapy. Among the available assays, Liquid Chromatography coupled with UltraViolet or Electrospray Tandem Mass Spectrometry methods (LC/UV or LC/ESI-MSMS) are the most accurate and specific ones. A reliable alternative is represented by immunoassays, which offer the opportunity to minimize sample pre-treatment, thus reducing the time between drawing blood sample and measuring the drug concentration, an important aspect in high-throughput analyses. Despite this, a limitation in the use of immunoassays for therapeutic drug monitoring is the lower specifity compared with the chromatographic methods when analysing structurally-related drugs.

New insights to optimize mTOR inhibitors regimens seem to be offered by the evaluation of CYP450 3A activity by using the probe drug approach. To such purpose, there are a number of major probe drugs used for *in vivo* studies including: midazolam, cortisol, lidocaine, nifedipine, dextromethorphan, erythromycin, dapsone and alfentanil.

The aim of the present paper is to report the most recent knowledge concerning this issue, supplying a critical and comprehensive review for whom are involved both in the clinical and analytical areas.

Keywords: Immunoassays, LC/UV, LC/MS, mTOR inhibitors, pharmacodynamics, pharmacokinetics, Therapeutic Drug Monitoring.

1. SCENARIO

The introduction of immunosuppressant drugs in the post-transplant treatment has improved both graft and patient survival rates. Among these pharmacological agents, mammalian Target Of Rapamycin (mTOR) inhibitors represent a new class of drugs more and more used in the immunosuppressive protocols. The main reason addressing to the choice of them is the good efficacy associated with a reduced toxicity [1]. The mTOR inhibitors have proven their efficacy and safety in numerous studies and are used either de novo or as a substitute in the follow-up after renal transplantation [1]. Conversion to mTOR inhibitors may benefit heart transplant recipients, particularly those with Calcineurin Inhibitors (CNIs) nephrotoxicity, in whom the concomitant reduction or withdrawal of CNIs could improve renal function. mTOR inhibitors provide effective immunosuppression after heart transplantation and their use has the potential to ameliorate renal dysfunction by allowing CNIs toxicity minimization. Further studies and long-term follow-up are required to confirm the impact of mTOR inhibitors in heart transplant recipients [2]. Both Everolimus and Sirolimus also seem to have a protective effect against the onset of graft vasculopathy [3]. Sirolimus is safe and may improve outcome in selected patients after liver transplantation [4].

Owing to the immunosuppressive effect, mTOR inhibitors can be used in the therapy of refractory autoimmune rheumatic diseases [5]. In fact, Sirolimus has been proposed as a new therapeutic option for the treatment of systemic lupus erithematosus [6]. In addition, Sirolimus offers a potential approach for the treatment of another immunologic disease, the Sjogren's syndrome; a recent study has shown success in animal models although human studies have so far failed to accomplish clinical endpoints, very likely due to inadequacies in study design [7]. However, it has been reported that Sirolimus inhibited the clinical and histopathologic incidences and severity score of rheumatoid arthritis in a dose-related manner and it was able to maintain the inhibitory effect on histopathologic changes after two weeks of the last dose, in contrast with cyclosporine showing a rebound of the disease state after discontinuation of the drug [8].

Recently, mTOR inhibitors offer a window into the care of a variety of human cancers. Rapamycin was shown to inhibit the growth of several murine and human cancer cell lines in a concentration-dependent manner. It is under investigation its use in the treatment of glioma, rhabdomyosarcoma, medulloblastoma, prostate, pancreatic and breast cancer [9]. A similar anticancer activity has been reported also for Everolimus [10].

Finally, this class of pharmacological agents has been proposed as adjuvant for the therapy of atherosclerosis. This is based on the autoimmune origin of this pathologic condition. In particular, Everolimus seems to favourably influence the atherosclerotic process by affecting the recruitment of monocytes into early lesions. This effect could be believed paradoxical because of the hyperlipidemia mTOR inhibitors-related, but several ongoing researches address towards this hypothesis [11-13].

2. PHARMACOLOGICAL ASPECTS

2.1. Pharmacokinetics

Pharmacokinetic properties of Sirolimus have been determined following oral administration in healthy subjects and patients. Fol-

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1389-2002/11 $58.00+.00

lowing administration of oral solution of Sirolimus, the mean times to peak concentration (t_{max}) of Sirolimus are approximately 1 hour and 2 hours in healthy subjects and renal transplant patients, respectively. The bioavailability of Sirolimus is low and estimated to be approximately 14% after the administration of oral solution. In healthy subjects, the mean bioavailability of Sirolimus after administration of the tablets is approximately 27% higher than the oral solution. Sirolimus concentrations following the oral administration in stable renal transplant patients are dose-proportional between 3 and 12 mg/m^2. In healthy subjects, Sirolimus was absorbed more slowly when administered after a high-fat meal than when administered after fasting, as shown by statistically significant reductions in peak concentration (C_{max}) and the ratio of C_{max} to the area under the concentration-time curve (AUC), and lengthening of the time to peak concentration; anyhow, the oral availability of Sirolimus is not uniform when administered with a high-fat meal and the geometric mean ratio of the fed/fasting AUC values was 1.35, with a 90% confidence interval of 1.26 to 1.46 [14]. The mean (± SD) blood-to-plasma ratio of Sirolimus was 36 ± 18 in stable renal allograft patients, indicating that the drug is extensively partitioned into formed blood elements. Sirolimus is extensively bound (approximately 92%) to human plasma proteins, mainly serum albumin (97%), α_1-acid glycoprotein, and lipoproteins. Sirolimus is a substrate for both CYP3A4 and P-glycoprotein (P-gp). Sirolimus is extensively metabolized in the intestinal wall and liver and undergoes countertransport from enterocytes of the small intestine into the gut lumen. Sirolimus is extensively metabolized by O-demethylations and/or hydroxylations. Seven major metabolites, including hydroxy, demethyl, and hydroxydemethyl, are identifiable in whole blood. Some of these metabolites are also detectable in plasma, feces, and urine. Sirolimus is the major component in human whole blood and contributes to more than 90% of the immunosuppressive activity. After a single dose of ^{14}C-Sirolimus oral solution in healthy volunteers, the majority (91%) of radioactivity was recovered from the feces, and only a minor amount (2.2%) was excreted in urine. The mean ± SD terminal elimination half life ($t_{½}$) of Sirolimus after multiple dosing in stable renal transplant patients was estimated to be about 62 ± 16 hours [14-17].

Everolimus pharmacokinetics has been characterized after oral administration of single and multiple doses in both healthy subjects and patients. After oral dosing, peak Everolimus concentrations occur 1 to 2 h post dose and Everolimus C_{max} and AUC are dose proportional. After a high-fat meal, t_{max} was delayed by a median 2.5 hours, and C_{max} was reduced by 50%. Overall absorption, however, was not affected by food as the fed/fasting AUC ratio was 0.99 (0.83-1.17) [18,19]. Plasma protein binding was approximately 74% in healthy subjects. The drug reached steady state within 4-7 days and has an elimination half-life of 16-19 hours [20]. Everolimus is the main circulating component in blood. Everolimus is a substrate of CYP3A4 and P-gp. The main metabolic pathways identified in man are monohydroxylations and O-dealkylations. Two main metabolites are formed and none of them significantly contribute to the immunosuppressive activity. After a single dose of radiolabel Everolimus given to transplant patients, the majority (80%) of radioactivity is recovered from the feces and only a minor amount (5%) is excreted in urine [21,22].

2.2. Pharmacokinetics in Specific Populations

Compared with the values in the normal hepatic function group, the patients with hepatic impairment have higher mean values for AUC of both mTOR inhibitors with no statistically significant differences in mean C_{max}. The maintenance dose should be reduced by approximately one third in patients with mild-to-moderate hepatic impairment and by approximately one half in patients with severe hepatic impairment. Therapeutic Drug Monitoring (TDM) is necessary in all patients with hepatic impairment.

Table 1. Main Drugs Interfering with Metabolism of mTOR Inhibitors

Inhibitors CYP 3A4	Inducers CYP 3A4
Bromocriptine	Atorvastatin
Oral Contraceptives	Carbamazepine
Clarithromycin	Phenobarbital
Danazol	Phenytoin
Diltiazem	Rifabutin
Doxycycline	Rifampin
Erythromycin	
Fluconazole	
Isoniazid	
Ketoconazole	
Metoclopramide	
Nicardipine	
Norfloxacin	
Verapamil	
Voriconazole	

The effect of renal impairment on the pharmacokinetics of both immunosuppressant drugs is low. In fact, there is minimal (2.2%) renal excretion of the drugs or their metabolites in healthy volunteers and their doses need not be adjusted in patients with renal impairment.

2.3. Drug-Drug Interactions

Drug-drug interactions are common occurrence for both mTOR inhibitors. In this pharmacokinetic aspect both CYP3A4 and P-gp play a key role. Oxidative drug metabolism by CYP enzymes is a major pathway for drug biotransformation. Among numerous CYP enzymes, CYP3A4 is the isoform that is most important for human drug disposition. It is the most abundant isoform in the liver and among CYP substrates it is responsible for metabolizing more than 50% of drugs currently administered [23]. P-gp, a transmembrane transporter, is present in the endothelium of several tissues, such as brain, lung and kidney. P-gp expression serves as a protective mechanism against the *stimuli* of cytotoxic agents, heat shock, irradiation and genotoxic stress. P-gp also transports immunosuppressive agents, such as cyclosporine, tacrolimus, sirolimus and everolimus [24,25]. P-gp is an ATP-dependent efflux pump associated with the multidrug resistance phenomenon. It is encoded by two genes MDR1 and MDR3 and the role of MDR3 P-gp in multidrug resistance has been proposed by several studies [26]. Sirolimus increases P-gp level whereas everolimus does not [27]. Unlike CYP metabolic system, in women P-gp is less expressed than in men and this could explain sex-related differences in the disposition of numerous drugs [23].

Considering such aspects, inhibitors of CYP3A4 and P-gp enhance blood concentrations with potential increase of toxicity risk, while inducers of CYP3A4 and P-gp decrease concentrations and consequently reduce the efficacy of the two drugs (see Table 1). The concomitant inducing or inhibitory effect of coadministered drugs on CYP3A4 and P-gp can be synergistic and dramatically modify blood concentrations of Sirolimus and Everolimus [28]. It is noteworthy to underline that the entity of the drug-drug interactions can be different in healthy volunteers or in patients. In fact, in previous studies, findings obtained in the formers were only partially or not at all confirmed in the latter [29-31].

Furthermore grapefruit juice reduces CYP3A4 activity and then it must not be drunk or used carefully during mTOR inhibitors treatment. On the contrary, John's Wort (*hypericum perforatum*) induces CYP3A4 and P-gp and there is the potential that its use in patients receiving mTOR inhibitors leads to a reduction of their blood concentrations [32].

2.4. Pharmacodynamics

mTOR inhibitors block T-lymphocyte activation and proliferation that occurs in response to antigenic and cytokine (Interleukin(IL)-2, IL-4, and IL-15) stimulation by a mechanism that is distinct from that of other immunosuppressants. They also inhibit antibody production and in cells bind to the immunophilin, FK Binding Protein-12 (FKBP-12), to generate an immunosuppressive complex. Such FKBP-12 complex has no effect on calcineurin activity. Instead, this complex binds to and inhibits the activation of the mammalian Target Of Rapamycin (mTOR), a key regulatory kinase (Fig. **1**). This inhibition suppresses cytokine-driven T-cell proliferation, inhibiting the progression from the G1 to the S phase of the cell cycle. In mammalian cells, TOR exists in the cytosol as a component of at least two distinct multi-molecular complexes: TORC1 (TOR-raptor-GβL) and TORC2 (TOR rictor-GβL) [33]. In general, raptor-containing TOR complexes (TORC1) are rapamycin-sensitive, whereas rictor-containing TOR complexes (TORC2) are rapamycin-insensitive [33]. However, in some cell types (including podocytes), the prolonged exposure to Sirolimus (24 h or more) may inhibit TORC2 assembly [34]. TORC1 promotes G1-phase progression and proliferation through multiple molecular mechanisms. In contrast to TORC1, TORC2 regulates growth factor signalling, cell survival and cytoskeletal reorganization, but the factors promoting its activation are not well defined [17].

2.5. Tolerability

The risk of adverse drug reactions reported with the use of mTOR inhibitors in organ transplant recipients is variable [35,36]. The most common side effects reported in clinical trials of transplant patients were myelosuppression (especially anaemia), impaired wound healing, hyperlipidemia, proteinuria and serious infection [35]. The incidence of side effects in non-transplant patient populations could be lower [37].

Both experimental animal and clinical data suggest that adverse reactions and the severity of their toxicity are correlated with drug concentrations [38]. Similarly, the widely described absence of renal impairment with mTOR inhibitors [39,40] was not found in two studies in whom high blood levels of Sirolimus were associated with serious renal dysfunction [28,41].

3. THERAPEUTIC DRUG MONITORING (TDM)

The relationship between the activity of a drug and its concentration in body fluids was first evidenced for quinacrine by Marshall in the 1940s [42] and became a routine clinical practice for immunosuppressants since the 1980s, with the introduction of Cyclosporine A [43]. The existence of such relationship can be considered the basis for an effective Therapeutic Drug Monitoring (TDM), defined as the measurement of a drug or its metabolite(s) concentration in a body fluid aimed to optimize the dose regimen [44]. TDM is an example of clinical relapse of the activity of a pharmacological laboratory, but its use requires that: i) the concentration measured in the body fluid is undoubtedly related to the concentration at the receptor; ii) the concentration at the receptor can be related to the effect; iii) the drug-receptor binding is reversible, so that drug exerts the effect for the period during which it can be detected; iv) there is a narrow margin between the concentrations associated to therapeutic and toxic effects,

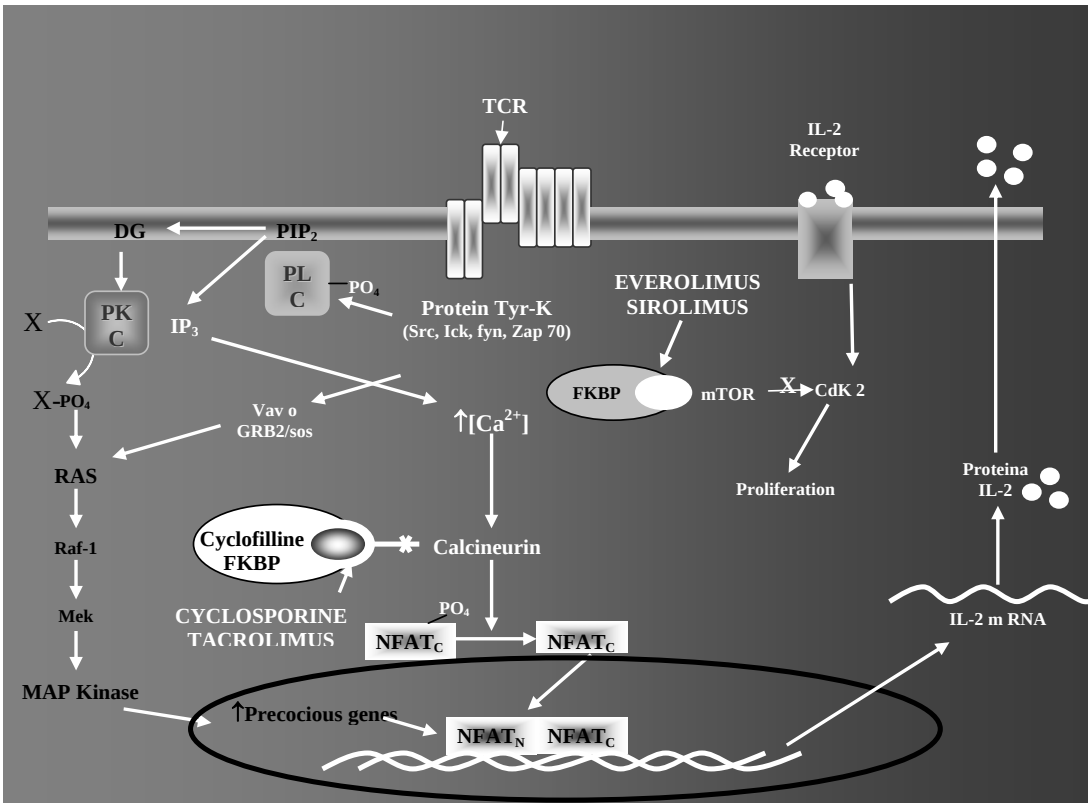


Fig. (1). Mechanisms of action of main immunosuppressant drugs.

tions associated to therapeutic and toxic effects, respectively. If such premises are satisfied TDM is effective and appropriate, since the clinician could use the results of the analysis for deriving the more proper variation in the dose regimen [44]. Otherwise, if there is a marked difference between "effective" and toxic doses or if a clinical marker can be used to state the effect of the administered dose (blood pressure, prothrombin time etc.), TDM is not so clinically relevant or even redundant. TDM is not simply a drug concentration determination, but a complex process which starts with a clinical question "The patient does not respond to therapy, is it attributable to inadequate dose?". In order to respond to such question a sampling strategy has to be developed, i.e. to choose: the marker(s) to detected, the body fluid to sample, the number of measurements to perform and, finally, the correct way to adjust dose regimen on the basis of the obtained results. Before considering such aspects in details, it must be stressed that therapeutic ranges represent a guide and not limits in which all patients must be forced. Really, "target range" is a more appropriate term, since a patient could need a plasma concentration higher than the upper therapeutic range limit (or conversely below the lower limit) to produce the desired effect.

Blood, urine, saliva and hair are in principle suitable for TDM, but blood is very often the matrix of choice. Hair offers the possibility of a wider temporal window with respect to the other matrixes (months vs. hours), but such property is not particularly useful for TDM and, really, the use of hair is limited to long-term monitoring of patients in psychiatry [8]. The use of urine is limited by the need of normalizing drug levels with respect to the degree of matrix concentration and the general hydration state. If the marker's saliva:plasma concentration ratio is constant and salivary pH does not influence marker's stability, TDM can be performed by sampling saliva. However, plasma, serum or whole blood represent the matrixes of choice for TDM, even if the sampling is invasive. Whole blood is preferred to plasma or serum when drugs are concentrated in red cells, as for TDM of immunosuppressants [45-47]. When using plasma or serum, care must be taken with anticoagulants, since they can interfere with the drug itself or with the assay system. In the specific case of mTOR inhibitors ethylenediamine-tetracetic acid (EDTA) is the anticoagulant of choice, since it allows minimizing clotting problems [48]. The total drug concentration detected in plasma (free plus protein-bond) can vary with the protein content, since they can bind considerable amounts of the marker [44]. In such cases the free drug concentration should be measured only (this can be performed *in vitro*, determining the drug concentration in a plasma ultrafiltrate, or sampling an *in vivo* ultrafiltrate). If plasma concentration is directly proportional to the concentration at the specific drug receptor (i.e. to the amount of administered drug that is exerting an effect), the quantification of marker plasma level can be used to discriminate between therapeutic and toxic doses and to define the pharmacokinetic and pharmacodynamic parameters.

The choice of the sampling time(s) is crucial for an effective TDM, especially for those drugs with brief half-lives [44]. Sampling must be done when the concentration of the marker (drug or its metabolite) is at the steady-state and the effects of the last variation in the dose have been allowed to stabilise. The time for the reaching of the steady-state depends on the elimination half-life and, normally, at least four half-lives have to elapse before monitoring the effects of a dose variation. Moreover, TDM is performed measuring the steady-state trough concentration, immediately prior to the next oral dose.

3.1. TDM of mTOR Inhibitors and Clinical Aspects

Since both immunosuppressive activity [50,51] and adverse effects [39,52,53] of Sirolimus have been related to its concentration, guidelines for its monitoring [54] and recommendations for its target concentrations after kidney transplantation [55] have been proposed. Whole blood was identified as the biological matrix for therapeutic drug monitoring because rapamycin is primarily partitioned into erythrocytes (94.5%) [15]. Drug half-life time, estimated in healthy volunteers, is 66.3 ± 18.8 h [56] and its rate of absorption depends on dosing forms and practice of ingestion with or without food [15]. A poor relationship between Sirolimus dosage and blood concentrations are reported [45], as a consequence the pharmacological effects have to be related to blood concentration indices, such as 24-hour area under the concentration-time curve (AUC_{0-24}) or trough concentration (C_0) [57,58].

Everolimus is characterized by a narrow therapeutic index [21,59] and bounds to red cells in a percentage higher than 75% at therapeutic concentrations [21], but blood levels may be affected by hepatic insufficiency and/or ethnicity [60,61]. The C_0 is recommended for TDM, since it correlates well with AUC and clinical outcomes [62,63]: C_0 less than 3 μg/l has been associated with high incidence of acute rejection, while levels higher than 8 μg/l have been related to increasing incidence of thrombocytopenia and dyslipidemia [64,65].

TDM of mTOR inhibitors is successfully performed using immunoassays and liquid chromatographic-based methods (with both UV (LC/UV) and mass spectrometric (LC/ESI-MS) detectors), each with its own peculiarities and limitations.

4. ASSAYS FOR TDM OF mTOR INHIBITORS

4.1. Immunoassays

Immunoassays represent a powerful tool for routinely and high-throughput analyses, since quantitative determinations can be obtained with sufficient accuracy and reproducibility. Moreover, tests are normally rapid and do not require great sample pre-treatment, so the time between drawing the sample and obtaining the analytical determination is reduced to the minimum.

All immunoassays are based on the reaction between the drug (antigen) and a specific antibody; the resulting antigen/antibody complex is, then, able to generate a detectable signal (or, alternatively, it reacts with an *ad hoc* reagent). The test system normally contains a fixed amount of labelled drug (that competes with the drug present in the sample for binding to the antibody), so that the signal generated by labelled antigen enable results to be compared with a calibration curve, for quantitative results. Since the binding sites are the same, the number of labelled drug molecules bound to the antibody is inversely proportional to the number of unlabelled drug molecule. After the immunoreaction takes place, there could be the need to separate the bounded drug molecules from the reaction mixture (that contains non-bounded molecules). Consequently, immunoassays are divided into two groups, homogeneous and heterogeneous. The latter requires the separation of the antigen-antibody complex; while, the former do not requires further purifications.

In the specific case of mTOR inhibitors, the Microparticle Enzyme ImmunoAssay (MEIA) on IMx® analyzer by Abbott Laboratories was firstly proposed for Sirolimus' TDM as an alternative to liquid chromatography based methods [66]. MEIA is an evolution of a homogeneous based method, in which particles of latex or similar present the drug conjugated onto them: the antibody presents two binding sites, when it is added to the test tube and reacts with the coated drug molecules, a bridge between them is formed, which results in agglutinates production. Such reaction competes with the drug-antibody binding, so the degree of agglutination is inversely proportional to the amount of drug present in the sample.

From the very first results MEIA on the IMx® allowed a rapid determination of Sirolimus with sampler saver: the test requires 150 μl of blood sample and is completed within two hours - the sample pre-treatment is reduced to zero [66]. The assay is based on the analysis of 5 non-zero calibrants with Sirolimus concentrations in the range 3-30 ng/ml, plus an analyte free calibrator. Since its first commercialization, the Sirolimus immunotest was first updated,

switching to a nonzero mode 1 calibrator mode (6 ng/ml instead of a zero 1 calibrator mode) [66], then the source of pure Sirolimus reference material used to prepare calibrators was changed [62], thus requiring new validation studies [66,67].

Briefly, a validation study is aimed to assess both the reliability and reproducibility of a particular method used for quantitative determination of analytes in a biological matrix. To validate an analytical methods accuracy, precision, selectivity, sensitivity, reproducibility, and stability have to be determined. According to the Food and Drug Administration Guidelines [68] for a replicated set of measurements:

- accuracy describes the closeness of the mean results to the true value; a minimum of five determinations per concentrations have to be performed and at least three concentrations has to be considered; method is accurate if the mean value is within 15% of the true value, and it does not deviate by more than 20% at the lower limit of quantification (LLOQ);
- precision represents the closeness of the single measure when multiple aliquots of a single homogeneous batch of the considered biological matrix are analysed; at least five determination per concentrations have to be analysed, obtaining precisions (in terms of CV%) not exceeding 15% (20% at LLOQ) at each concentration level;
- selectivity reflects the ability of the analytical method to discriminate between the analyte and interfering compounds present in the biological matrix; at least five blank samples have to be analysed and quantified, obtaining a fictitious analyte concentration below the LLOQ;
- sensitivity of an analytical method can be expressed in terms of limit of determination (LOD) and LLOQ. The former represents the lowest concentration level statistically different from a blank; the latter is the level above which quantitative results may be obtained with a specified degree of confidence. Both LOD and LLOQ can be calculated through the quantification of at least five blank samples: considering the SD associated to the fictitious analyte concentration obtained for the set of blanks, LOD is 3*SD, while LLOQ is 5*SD;
- stability is function of the storage conditions and it has to be determined in order to define if sampling, storage and even purification procedures are able to avoid analyte degradation; both short-term and long-tern storage conditions have to be tested, as well as evaluation of analyte stability in stock solution.

Moreover, the FDA Guidelines specify that recovery of the analyte from the biological matrix has to be calculated comparing the detector response recorded for samples prepared in the biological matrix with the signal obtained for the same samples prepared in pure solvents; at least three concentrations have to be considered.

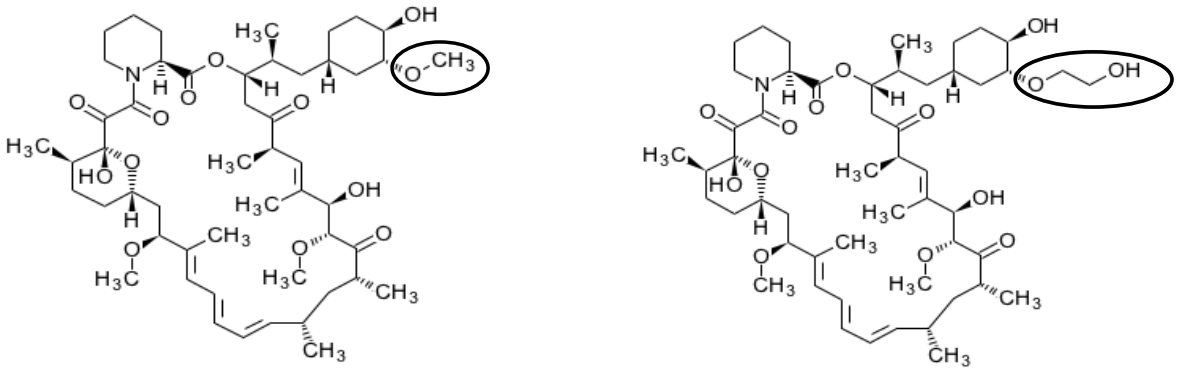
Assessing the robustness of Sirolimus quantification by MEIA includes the determination of validation parameters, as specificity, precision, accuracy, limits of detection and quantification (LOD and LLOQ, respectively), and a comparison of immunotest performance with respect to liquid chromatography-based methods [66,67,69-74]. In particular, specificity of MEIA was firstly investigated with respect to Sirolimus metabolites and, recently towards drug' analogues, especially Everolimus [75-77]. Results of a multicenter evaluation of the new MEIA kit performance have been published in 2006 [74]: the study involved eight European sites, located in England, Scotland, Germany, Italy, France, Spain and Austria. Evaluation of MEIA performance included protocols for precision, limits of detection and quantification, antibody specificity, linearity, interferences (both from endogenous and exogenous substances) and comparison of the immunotest with respect to HPLC-based methods. Results of the multi-center study are consistent with previous reports [66,78]: test response showed linearity within the dynamic range (0-30 μg/l), with percent recovery between 91% and 125%; LOD and LLOQ ranged from 0.50 to 0.75 μg/l and 1.3 to 1.9 μg/l, respectively; precision, expressed in terms of CV% and calculated using quality control samples from 5 to 22 μg/l, ranged from 5.7% to 12.6%; endogenous compounds investigated gave less than 10% interference in MEIA quantification, the only significant relationship was observed with hematocrit values in the range 25%-45%; none of the investigated exogenous compounds resulted quantitable in free-Sirolimus samples [74]. Interestingly, as regards the method comparison with HPLC-based procedures the authors reported a general positive bias by MEIA (approximately 25%), with higher values for MEIA/HPLC-MS than MEIA/HPLC-UV, but the nature of such difference is unclear. A cross-reaction of MEIA with Sirolimus metabolites is also reported, ranging from 6% to 63%. So, data reported indicate that the latest MEIA kit overcomes the limits of the previous version. However, care must be taken in routine TDM and highly specific methods still remain the best choice to overcome errors in Sirolimus quantification due to cross-reaction with metabolites.

An alternative to MEIA a homogenous immunoassay has been developed for Sirolimus [79], based on Cloned Enzyme Donor ImmunoAssay (CEDIA), in which the binding to an antibody is used to influence the activity of an enzyme: when the substrate is present in the sample, the enzyme activity is increased [80]. Data obtained with the homogeneous immunotest showed a mean bias of 20.4%, better than that obtained with MEIA, but the same authors conclude that "neither immunoassay could be reasonably considered to have specificity for the parent drug" [79].

Immunoassay determination of Everolimus is based on a Innofluor® Certican Fluorescence Polarization ImmunoAssay (FPIA) commercialized by Seradyn Inc. FPIA is based on the "Strokes' shift", so the detection wavelength is moved from that used to excite the fluorophore to that at which it emits the absorbed light; the fluorescent molecule can be generated by a label enzyme [816]. According to manufacturer's specifications, 600 μl of whole blood aliquots (in EDTA) were added with 700 μl of methanol and 100 μl of Innofluor® Certican® precipitation reagent; after vortexing and centrifugation, aliquots of the supernatant are loaded into the TDx® carousel for the subsequent analysis. Calibration curve is obtained from the analysis of five calibrants (in the range 2.59-39.78 ng/ml) plus an Everolimus free-calibrator; moreover, three quality control samples (in the range 3.95-22.05 ng/ml) are used for daily quality control and to verify FPIA accuracy and precision. In the manufacturer's specification an analytical sensitivity of 0.80 ng/ml is reported, calculated as the concentration at two standard deviations from the mean rate count signal of the TDx® Everolimus calibrator at 0 ng/ml. Accuracy, intra- and inter-day precision (expressed as CV%) ranged between -5.8% and -11.4%, 6.9% and 10.5%, 7.7% and 10.4%, respectively.

As for Sirolimus quantification by both MEIA and CEDIA, the Everolimus determination by FPIA has been investigated as regards its accuracy, precision and specificity [82-85]. While accuracy and precision parameters are within acceptable criteria (varying in the ranges ≤6%-15% and ≤13%-15%, respectively), the FPIA generally overestimate Everolimus concentration with respect to a HPLC/MS method, with a mean bias of 24% [84-86]. However, also for FPIA its cross-reactivity with Everolimus metabolites is the real problem: almost all authors obtained a non-negligible cross-reaction that can be considered the main source of drug overestimation by immunotest. As for Sirolimus MEIA and CEDIA, as a consequence of FPIA cross-reactivity with drug metabolites clinically importance errors can derive [84,86].

The need of more specific analytical determination is even more critical if cross-reaction of the immunotest with substrate-related drugs is considered. In the specific case of Sirolimus and Everolimus this represents a critical aspect. Due to the almost superimposable chemical structures – they differ only for a 2-hydroxyetil chain at position 40 of the macrolide ring (Fig. **2**), a



a **b**

Fig. (2). Chemical structures of sirolimus (**a**) and everolimus (**b**). Differences in the chemical structures are evidenced in circles.

region not crucial for antigen-substrate recognition – a cross-reactivity of the immunotests is reasonably. Despite the similar mechanism of action, the two molecules are characterised by different pharmacokinetics [87] and there could be the need of switching patients from a drug to the other. In such cases is still possible to use MEIA and FPIA for an effective drug monitoring? Or is it necessary to use specific chromatographic-based determinations to discriminate between the two molecules, thus avoiding overestimations of drug blood levels? Such aspect was firstly investigated by Baldelli *et al* [85], Bouzas *et al*. [75,77] and Pieri *et al.* [76] by determining Sirolimus whole blood concentrations using the Innofluor® Certican® assay and/or vice versa (i.e. quantifying Everolimus using the Abbott IMx® assay). A high cross-reactivity was found both for MEIA and FPIA, concentration-dependent for the former immunoassay [75-77,85]. In a study on ninety-five samples from 53 renal transplanted recipients on Sirolimus treatment and one-hundred samples from 28 renal transplanted recipients on Everolimus treatment correlation degrees between drugs concentrations measured in by both MEIA and FPIA were so high that the Authors suggested that "both assays could mutually represent a reliable and accurate alternative to be considered in the case of unavailability of the specific-drug immunoassay" [76].

Almost all the papers aimed to test the efficacy of immunoassays kits conclude that despite precise, reproducible, sensitive and accurate quantifications can be obtained, specifity still remains the critical point and in particular situations, where difficult clinical issues arise, it is advisable to carry out the TDM with highly specific chromatographic-based methods.

4.2. High Performance Liquid Chromatography/ Ultraviolet Detection

High performance Liquid Chromatography coupled with Ultraviolet Detector (HPLC/UV) has been the technique of choice for Sirolimus TDM since early '90 [88-90]. HPLC/ UV ensured the required accuracy and precision; sensitivity and also specificity of the technique has been increased in the most recent studies by improving purification procedures [91-96]. Due to the relatively low concentrations of Sirolimus used in therapy (in the range 0.01-0.3 mg/Kg, [91]) and considering the combinations of immunosuppressants and other drugs administered to patients, specificity and sensitivity of the assay used for TDM purposes are crucial point for an effective drug determination and, consequently, dose regimens.

The first HPLC/UV application aimed to quantify Sirolimus levels in rabbit heterotropic heart transplanted model suffered of low sensitivity, due to the low drug percentage recovery from the haematic matrix (low than 35%) [97]. The first studies on routine clinical monitoring with sufficient sensitivity (and recovery of 95%) were based on internal standards with a different chemical nature with respect to Sirolimus [90,98]. Drug purification from the biological matrix and the use of the most suitable internal standard (I.S.) represent two essential points in the development of a HPLC-based quantification method. In principle, when the HPLC is equipped with an UV detector the quantification could be based on the Bear-Lambert Law [99]. Nevertheless, also for HPLC/UV a quantification based on a calibration curve is preferred when the analyte is present at low concentrations in a complex matrix, as in the case of mTOR inhibitors. The use of calibrators prepared in the biological matrix at know analyte concentrations and purified according to the same procedure used for "real" samples (i.e. samples from transplanted recipients) does not ensure the minimization of all errors that could occur during sample treatment, thus requiring the use of a "normalizing agent", the internal standard: each sample - calibrators, quality controls and "real" samples - is added, prior of purification began, with known amounts of a molecule structurally-related to the analyte of interest, so that each error and/or variation in the analytical procedure will have a similar impact on both analyte and internal standard concentrations. If calibration curves are constructed in a relative way, by reporting the ratio between analyte' and internal standard' areas vs. nominal analyte concentration, a normalization from any error occurring during the whole analytical procedure will be obtained. Such normalization, of course, will be as much effective as the more similar the chemical natures of the analyte and the internal standards will be: stable isotopes labelled are the most suitable internal standards, followed by analyte' structural analogues and, finally, by "analyte-related molecules" (i.e. molecules containing some functional groups similar to those present in the analyte under study). Considering such aspects, since early 2000' literature studies aimed to quantify Sirolimus by HPLC/UV have involved a Sirolimus analogue as internal standard, the desmethoxySirolimus (DMR) [91-96], which has allowed a better and simpler analytical determination with respect to other internal standards, as for example β-estradiol-3-methyl ether proposed by Napoli and Kahan in 1994 [90]. Due to the strict analogy between Sirolimus and Everolimus, DMR has been used as internal standard for the latter drug quantification, too [85,100-102]. Recently, ascomycin has been used as internal standard for simultaneous Everolimus and Sirolimus quantification [103,104]. HPLC/ UV procedures reported in literature for Sirolimus and/or Everolimus quantification differ as regard drug(s) purification procedure and chromatographic elution, improved to maximize drugs recovery from the haematic matrix and eliminate interfering peaks.

The procedure proposed in 1994 by Napoli and Kahan [90] for Sirolimus purification involved the use of a darkened hood, wrapping tubes and brown glass to protect against photolysis and a liquid-liquid extraction with *ter*-buthyl methyl ether followed by ethanol: despite its complexity, the procedure allowed a 96% recovery

from 1 ml of blood. The subsequent HPLC elution involved two reverse-phase C_{18} columns in tandem and 85% methanol/water mixture as mobile phase [90]. Since then, Sirolimus purification procedure has been simplified, without lack of sensitivity. The most used off-line Sirolimus purification procedures, schematized in Table 2, involve the initial blood protein precipitation followed by a liquid-liquid extraction, eventually coupled with a solid phase extraction. Despite analyte' recovery in the range 61%-106%, the procedures are time consuming, requiring up to 4 hours to be completed [89-96].

Considering the chemical nature of Sirolimus and Everolimus - lipophilic macrolide lactones – the chromatographic elution is performed by reverse-phase C_{18} or C_8 columns (the latter is mainly used for Sirolimus, while the second for Everolimus), usually kept at 50°C to improve performances; the UV detector is settled at 278 nm for both Sirolimus and Everolimus; as far separation, HPLC/UV methods reported in literature are mainly based on isocratic elution with different solvents: for Sirolimus 90% methanol [95], methanol/acetonitrile/water mixture, at different composition (68/2/30, 50/22/28, 30/36/34, 30/42/28, v:v) [91,104,94,96], 60%-65% acetonitrile [93,92], while lower acetonitrile (56%) percentages are used for Everolimus isocratic elution [85,100].

The HPLC/UV procedures show Lower Limit Of Quantification (LLOQ) close to the minimum therapeutic concentration - LLOQ in the range (2.5-6.5) ng/ml are reported [89-96] for Sirolimus, while quantification limits of 1 ng/ml [100] and 1.5 ng/ml [105,106] have been obtained for Everolimus. This aspect cannot be underrated, since values of LLOQ close to the minimum therapeutic range could mine the efficacy of the quantification in those samples when drugs are present at very low concentrations.

Another important aspect to be considered is the need of optimize the chromatographic elution, to have well-resolute peaks. HPLC/UV chromatograms give a bi-dimensional information, analyte' retention time (RT) and signal intensity; peak assignment is based on the RT, so the presence of interfering molecules, eventually co-eluting with the analyte of interest, can sensibly alter the quantification result. The possibility of co-elution is particular critical when more than one drug as to be monitored, as for simultaneous quantification of mTOR inhibitors, since the strict structural analogy between Sirolimus and Everolimus makes harder to achieve a complete peaks separation, i.e. resolution at the base-line.

In conclusion, specificity and sensitivity of the HPLC/UV procedures represent the main weaknesses, especially when the analytes are present in the haematic sample at very low concentrations. Even if a specifity greater than immunoassays can be achieved, time-consuming and somehow tedious purification procedures must be settled-up, so HPLC/UV remains the second most used technique for mTOR inhibitors quantification and the most recent applications are based on HPLC coupled with mass spectrometry.

4.3. High Performance Liquid Chromatography/Mass Spectrometry

High Performance Liquid Chromatography coupled with Mass Spectrometry (LC/MS) represents the technique of choice in the most recent applications aimed to quantify molecules present at low concentration in complex matrixes, as for mTOR inhibitors. A quantification method based on HPLC/MS is extremely specific, sensitive and accurate, run-times are shortened with respect to other HPLC-based methods and also purification steps can be less time consuming, since "more impure" samples can be analysed due to the high specificity and sensitivity of the mass analyser. Moreover, the introduction of the Electrospray Ionization (ESI) increased the number of chemicals that can be detected with mass spectrometry: ESI is a low-energy ionization technique, thus allowing the analysis of thermo-sensitive molecules and the minimization of in-source fragmentation processes [105]. Molecules eluting from the HPLC column (or directly injected into the mass spectrometer) and present as ions in the condensed phase, are vaporized into the ion source of the mass spectrometer; then ions are accelerated under high vacuum condition by applying an electric fields and subsequently separated on the base of their mass-to-charge ratio (*m/z*) by a magnetic field; finally, "ion packages", i.e. ions with the same mass-to-charge ratio, reach the detector to produce a recordable signal.

In principle an ESI-MS quantification can be based on the detection of whatever ionic form of the analyte under study, positively or negatively charged (ESI^+ or ESI^-, respectively), provided that such counter-ion can be added to the mixture used for HPLC elution (or for solubilising the analyte, if it is directly injected into the mass spectrometer) and that a stable ionic complex with the analyte is formed; moreover, the type of mass analyzer present in the spectrometer (ion trap or quadrupole) is also able to influence the choice of the counter-ion. When positive electrospray ionization is used, detection is based on the complex between the analyte and *n* molecules of positively charged counter-ions, $[M+nion]^{n+}$ (*n*, the number of counter-ions that will complex the analyte, depends on the number of protonable sites present into the analyte). Acidification and the addition of sodium, potassium or ammonium ions to the solvent mixture are typically used in positive electrospray ionization, thus, in the case of just one protonable site, the MS analysis can be based on the detection of the mass-to-charge ratio of the species $[M+H]^+$ (pseudomolecular ion), $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, respectively.

Depending on analyte's chemical nature, ionization technique and overall analytical conditions used, each molecule is able to generate a particular pattern of ionic species, that can be registered obtaining the so-called MS-*full scan* mass spectrum: it is considered the fingerprint of a molecule, since it contains the total ion current originated by the analyte as result of the ionization process. In order to improve sensitivity, selected ions from the total ion current can be monitored (Selected Ion Monitoring, SIM); alternatively, a precursor (or parent) ion can be isolated from the total current and further fragmented, by applying a supplementary voltage: obtained fragment ions can be detected all or in part, obtaining a MS-MS or MS-MS-MRM (Multiple Reaction Monitoring) analysis; alternatively, the process can be reiterated (tandem mass spectrometry, MS^n). It must be noted that multiple mass spectrometry stages involve the selection of a smaller and smaller fraction of the total ion current generated in the ionization process, and this represents an instrumental limitation to the increment of sensitivity achievable with tandem mass spectrometry; however, quantification methods are normally based on MS-MS, so such aspect is not critical.

To confirm the presence of the analyte under study in an "unknown sample" the MS-SIM or MS-MS mass spectra have to contain all and only the selected ions or the fragment ions; moreover, relative ion intensities recorded have to match those obtained in the analysis of a standard solution of the analyte and this results in an increased specificity (the possibility that an interfering compound gives rise to exactly the same fragmentation pattern of the analyte under study is extremely low) and sensitivity (since the signal-to-noise ratio is improved) of both MS-SIM and MS-MS analyses with respect to a MS-*full scan*.

In the case of mTOR inhibitors the use of low-energy ionization processes, as the electrospray is, minimize the possibility of drug underestimation, since, due to in-source fragmentation processes, the analyte could originate fragments with the same mass-to-charge ratios of metabolites. Moreover, the minimization of in-source fragmentations results in easy-to-interpret mass spectrum.

Streit and colleagues published the first HPLC/ESI-MS method for the quantification of Sirolimus and its major metabolites levels in whole blood in 1996 [106]: the assay was based on the purification of 1 ml blood through a C_{18} extraction column followed by the LC/ESI-MS analysis, focused on detection of the $[M+Na]^+$ ions

Table 2. Whole-blood Sirolimus Purification Procedures Reported in HPLC/UV Quantification Methods

Purification Procedure	%Recovery	Reference
1 ml blood + I.S.[a] + 1 ml 0.1M sodium carbonate + 100 µl methanol ↓ vortex – mixing + 10 ml *t*BME[c] } x 2 ↓ shaken, centrifuge dry under nitrogen stream ↓ +150*µlEthanol* x2 ↓ shaken, centrifuge recover the ethanolic layer dry under nitrogen stream + 100 µl 85% methanol ↓ shaken, centrifuge recover the clear supernatant liquids and inject	96%	[56][b]
2 ml blood + I.S.[a] + 3 ml 13.3 potassium carbonate + 5.5 ml diethyl ether ↓ shaken recover the organic layer and dry + 150 µl 70% methanol (mobile phase) + 100 µl hexane ↓ vortex, centrifuge, evaporate the hexane layer mobile phase layer injected	~100%	[55][b]
1 ml blood + I.S.[a] + 3 ml 0.1M sodium acetate + 7 ml 1-chlorobutane ↓ shake for 1 h at 250 shakes/min centrifuge for 10 min at 3000rpm place in a dry ice/methanol bath recover the organic layer and dry ↓ + 300 µl 70% methanol, vortex and centrifuge recover the supernatant for injection	88%-106.3%	[57][b]
1 ml blood + I.S.[a] + 4 ml extraction mixture[d] ↓ mix, centrifuge dry the organic layer in a concentrator + 0.5 ml 50%acetonitrile + 0.5 ml hexane ↓ mix, centrifuge, recover the hexane layer 200 µl of the sample extract used for injection	81.5%±4.3%	[58][b]
0.5 ml blood + I.S.[a] + 1 ml zinc sulphate 50 g/l + 1 ml acetone ↓ vortex, centrifuge for 5 min at 2600g recover the supernatant + 200 µl 100mM sodium hydroxide ↓ vortex, centrifuge for 5 min at 2600g recover the supernatant and dry under nitrogen + 150 µl 60%acetonitrile + 500 µl hexane ↓ vortex, centrifuge for 5 min at 2600g discard the hexane layer 50 µl of the sample extract used for injection	80.6%	[59][b]

(Table 2) Contd….

Purification Procedure	%Recovery	Reference
1 ml blood + I.S.[a] + 1.5 ml zinc sulphate 50 g/l + 1.5 ml acetone ↓ vortex, centrifuge for 5 min at 3000g recover the supernatant + 2 ml distilled water ↓ load on SPE extraction cartridge wash with 1.5 ml 70%acetonitrile, 500 µl hexane elute with 1 ml acetonitrile ↓ dry under nitrogen stream + 150 µl water/methanol/acetonitrile=40/30/30	61.1%±3.1%	[60][b]
condition the extraction cartridges (3 ml acetonitrile; 3 ml methanol; 3 ml distilled deionised water), using 2 ml/min flow mix 500 µl blood + I.S.[a] + 1.5 ml distilled deionised water + 500 µl acetone ↓ vortex + 4 ml zinc sulfate reagent[e] ↓ vortex and centrifuge recover the supernatant and add to the conditioned extraction cartridges wash with: (3 ml distilled deionised water) x2; 3 ml 75% methanol ↓ dry at high vacuum for 5 min elute with 2 ml 90%methanol ↓ dry at 45°C using an evaporator + 40 µl methanol for injection	101.8%	[61][b]
500 µl blood + I.S.[a] + 1 ml 5% zinc sulfate + 1 ml acetone ↓ vortex, centrifuge for 5 min at 4 °C recover the supernatant + 200 µl 100mM sodium hydroxide ↓ vortex, centrifuge recover the upper organic layer and dry + 150 µl 70% acetonitrile and vortex + 500 µl hexane ↓ vortex, centrifuge discard the hexane layer inject the sample extracts	96%-120%	[62][b]

[a]I.S., internal standard; [b]reference refers to numbered reference in the text; [c]*t*BME, *ter*-butyl methyl ether; [d]extraction mixture, 4 ml methyl ter-butyl ether/1-chloroethane/metahnol=45/45/10); [e]zinc sulfate reagent, 7.5 g zinc sulfate, 400 ml distilled deionised water, 100 ml methanol and 300 ml acetonitrile.

both for Sirolimus (*m/z* 936.6) and metabolites; a limit of quantification of 0.25 ng/ml (the HPLC/UV procedures have LLOQ in the range (2.5-6.5) ng/ml [89-96]), a recovery of 88%±26%, inter-assay variation of 19% at 1 ng/ml and of 9.3% at 15 ng/ml were obtained. The less elaborated extraction procedure allowed a faster quantification with respect to a HPLC/UV method; moreover, the developed procedure allowed simultaneous Sirolimus metabolites quantification.

Following studies have been focused on the quantification of Sirolimus ammonium ion, $[M+NH_4]^+$ at *m/z* 931.8 Da, using the MS-MS-MRM mode, based on the transition *m/z* 931.8 → *m/z* 864: pseudomolecular ion at *m/z* 931.8, chosen as precursor ion, is isolated and further fragmented, daughter ion at *m/z* 864.4 is selectively recorded [107-117]. In such studies, quantification of the internal standard is based on a similar transition: the DMR ammonium ion, at *m/z* 901.8, is used as precursor and daughter ion at *m/z* 834.4 is selectively recorded, by monitoring the transition *m/z* 901.8 → *m/z* 834.4.

The possibility of using an ionic complex different from the ammonium as precursor ion in developing an ESI-MS-MS method for Sirolimus quantification has been poorly investigated: few works are based on the Selected Ion Monitoring of the $[M+Na]^+$ ions, at *m/z* 936.6 for Sirolimus and at *m/z* 906.4 for DMR [118-122]. In 2005 a study was published [123], aimed to compare the performances of two LC/ESI-MS-MS-MRM methods, the first based on the sodiated adducts (transitions: *m/z* 936.5 → *m/z* 936.5, 904.4, 614.2, 582.2 for Sirolimus and *m/z* 906.4 → 874.3, 856.3, 733.3, 584.2 for DMR), the second based on the ammoniated species. The study involved the used of two different mass analyser: an ion trap mass spectrometer was used for setting-up the sodiated-based method, while a triple quadrupole mass spectrometer was used for the ammoniated complex detection. Almost superimposable results were obtained as regards LOD (0.7 ng/ml for the sodiated-based method, 0.5 ng/ml for the ammoniated-based method) and LLOQ (2.4 ng/ml for the sodiated-based method, 1.7 ng/ml for the ammoniated-based method), accuracy and precision, thus con-

firming the actual possibility to adopt the quantification of the sodiated species for routinely determination of rapamycin as an alternative to the commonly adopted method based on the ammoniated complex [123].

As reported, a review of literature evidences that DMR is largely used as internal standard for SIRO quantification by HPLC/MS, too. Really, when setting-up a mass spectrometry-based method a critical point to be accounted is the so-called "matrix-effect": when analytes are present in a complex matrix, interfering compounds coeluting with molecule(s) under-investigation can alter the efficiency of the ionization process. Tang and Kerbarle firstly evidenced such effect in 1993 for the ionization of organic bases [124] and since then almost all studies devoted to the optimization and validation of an HPLC/MS method have to take into account the matrix-effect. Matrix-effect can be minimized by improving purification and/or chromatographic separation (so that interfering compounds can be eliminated and/or eluted at different retention times) [125]. A recent study by O'Halloran *et al.* investigated the possibility to use the deuterium-labelled sirolimus (d_3-SIRO), concluding that it is able to minimize matrix-effect, ensuring a more accurate quantification with respect to DMR [126].

In the case of Everolimus LC/MS methods involve the detection of both positive and negative ions. In 1998 an ESI-MS-SIM method, based on the detection of the sodiated adduct at *m/z* 980.0, was published: a LOQ of 0.1 ng/ml was achieved and validation parameters were within acceptable criteria [127]. In 2000 a method based on the Atmospheric Pressure Chemical Ionization (APCI) operating in negative mode has been published: Everolimus quantification was performed by a MS-SIM based on the isolation and detection of the $[M]^-$ ion at *m/z* 957.6 [128]. The method was characterized by a LLOQ of 0.375 ng/ml, allowed the simultaneous detection of cyclosporine and, due to the optimization of a semi-automated 96-well solid-phase extraction system, results particularly suitable for high-throughput analyses and pharmacokinetic studies. In a subsequent study the authors switched to an ESI-MS-MS-MRM method, using the ammonium ion as precursor for Everolimus quantification (transition *m/z* 975.5 → *m/z* 908.5 was monitored), and a slightly lower LLOQ was obtained, using 0.3 ml of sample [129]. Moreover, the use of the liquid/liquid extraction allowed a marked improvement in sample throughput since less than 28 hrs were needed for the analyses of 384 samples. The same mass spectrometric transition was monitored by Salm and coll., involving different sample purification (protein precipitation followed by C_{18} solid-phase extraction): a LLOQ of 0.5 ng/ml, recovery from the biological matrix of 94.8±3.8% and validation parameters were within acceptable criteria were obtained [130].

The greater sensitivity and specificity of the LC/MS with respect to both LC/UV and immunoassays is confirmed by the lowest LOD and LLOQ of the mass spectrometry-based methods that are characterized by optimal accuracy, precision and reproducibility. Moreover, the possibility of on-line purification results in a reduced sample pre-treatment and time saving, aspect of particular importance in high-throughput analyses.

Another advantage of the mass spectrometry with respect to other assay/detectors is the possibility of simultaneously detect more analytes, without losing specificity, sensitivity or accuracy. In the case of TDM of immunosuppressants such aspect is extremely relevant, since patients are administered with different drugs, able to mutually interfere with absorption/distribution into the organism, so the possibility of simultaneously quantify almost all the immunosuppressants administered is of undeniable importance for optimal drugs prescription.

Summarizing, despite the initial high costs of the instrumentation and the need of expert technicians, the remarkable advantages of the LC/MS quantification make it the technique of choice for optimal TDM of immunosuppressants.

5. NEW INSIGHTS FOR OPTIMAL IMMUNOSUPPRESSIVE REGIMENS

Considering the small difference existing between therapeutic and toxic range of mTOR inhibitors, a critical aspect is represented by the need of optimal immunosuppressive regimen, in order to avoid both rejection risks and excessive immunosuppression. Such aspect is of great relevance especially for *de novo* transplanted patients, for which the possibility of predict the optimal oral dose to be administered could minimize organ rejection crisis.

In general, the oral dose to be administered to a patients depends on his ability to metabolize the active drug; mTOR inhibitors are metabolized by cytochrome P450 (CYP) system, in particular, by the CYP3A4 isoenzymes and their bioavailability is strictly related to individual metabolic activity of CYP3A4 [15]. There are many factors able to influence the enzymatic activity, among which drug-to-drug interactions, patients' gender, sex, life style (particularly smoking habit), general clinical picture and so on.

An interesting approach to define the metabolic activity is the "probe drug approach" [131], widely used in many clinical investigations, both for elucidating drugs metabolism and for pharmacogenetics purposes. The possibility of using probe drugs to evaluate the real-time activity of the CYP isoenzymes of interest is ensured by the fact that more than 90% of drugs used in therapies are metabolized by five CYP isoforms, CYP1A2, 2C9, 2C19, 2D6 and 3A [132,133], so the activity of each isoenzyme can be obtained by using specific drugs, namely "probe drugs", administered under controlled conditions. It is also advisable to strengthen the clinical importance of the "probe drug approach". In particular, the toxicity of flutamide, drug widely used in endocrine therapy for prostate cancer and mainly metabolized by CYP1A2, has been investigated by using the caffeine test which can be considered an effective probe of the activity of CYP1A2 and the obtained findings demonstrated that a decrease in CYP1A2 activity was involved in the onset of flutamide-induced hepatic injury. Moreover, the caffeine test provided a useful means to predict this adverse effect drug-related [134]. Similarly, it has been referred that the measurement of monoethylglycinexylidide (MEGX) concentrations, the main lidocaine metabolite forming via CYP3A4, could be a marker for evaluating the evolution of chronic hepatitis into cirrhosis. In fact, MEGX formation decreases as liver disease evolves, likely because of a reduction of the cellular functioning mass. Thus, MEGX test, used as probe of CYP3A4 activity, could integrate both the histological grading of chronic hepatitis and the clinical staging of cirrhosis [135].

The approach is even more valid if a population study is carried out, so that "reference" ranges of the metabolic activity can be derived and used to define the normal-, ipo- and iper-metabolizers. For each CYP isoenzyme the most suitable probe drug is chosen, considering that i)it has to be exclusively metabolized by the isoenzyme of interest; ii)the metabolic pathway has to be known; iii)it hasn't to be toxic and iv) a chemical standard has to be commercially available. Really, the "single probe drug" approach had a practical limitation: in general, patients are administered with multiple drugs, eventually metabolized by different isoenzymes families so there is the need of simultaneously determine the activity of all of them. Such limitation was overcome by the "cocktail approach" that consists in the administration of more probe drugs, one for each isoenzyme to be phenotyped [131,136-142]. Since both pharmacokinetic and pharmacodynamic interactions between probe drugs constituting the cocktail can occur, the overall analytical procedure used for enzymes activities evaluation has to be validated before widespread application [143]. Experiments performed during the validation procedure usually include the administration of both single and combination of the selected probe drugs and the comparison of the obtained isoenzymes activities, so that interactions between phenotyping probes can be highlight and corrections in the probes constituting the cocktail eventually made. Normally, plasma

or urine are the matrixes of choice for probes and metabolites detection [131,136-142,144].

Midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo [1,5-a][1,4]benzodiazepine) has been proposed as probe drug for CYP3A phenotyping [131,145]. 1'-Hydroxymidazolam is the main plasma metabolite and is used for enzyme activity determination: the ratio 1'-hydroxymidazolam/midazolam at 1 hour form probe drug administration is used for CYP3A phenotyping [146].

Analytical procedures settled-up for probe drugs detection are robust – the validation acceptable criteria are fulfilled – and recent applications based on the HPLC/tandem mass spectrometry ensure fast and sensitive quantifications, so the phenotyping approach is becoming a more and more suitable tool for optimal dose-regimen, with potential positive results in immunosuppressive therapies.

6. CONCLUSIONS

mTOR inhibitors represent a class of immunosuppressive drugs currently used in several therapies. In particular, they are utilized to prevent and/or treat graft organ rejection. Owing to their immunosuppressive activity, these pharmacological agents are also part of drugs administered in several autoimmune diseases. Besides these classical treatments, recently mTOR inhibitors have been proposed as new options in the therapy of some carcinogenic forms and atherosclerosis. Due to wide pharmacokinetic variability and the narrow difference between therapeutic and toxic ranges, a therapeutic drug monitoring is recommended. TDM can be performed by different analytical methods, among which immunoassays and liquid chromatography-based (both HPLC/UV and HPLC/MS) ones are the most used. Immunoassays are easy to perform and suitable for high-throughput analysis, but specificity still remain a weak point. HPLC/UV procedures have been widely used during the past years, but the most recent applications are based on mass spectrometric detectors, since high sensitivity and specificity are ensured and less time-consuming purification procedures can be carried out. At present, both for therapeutic drug monitoring and evaluation of drug pharmacokinetics HPLC/MS can be considered the gold standard analytical method.

Many efforts have been addressed to optimize immunosuppressive regimen and a promising perspective is represented by the "probe drugs" approach.

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Received: January 20, 2011 Revised: February 21, 2011 Accepted: February 22, 2011