Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 3323–3331
Received 15 April 2021; Accepted 05 May 2021

Measurement of Tacrolimus: A Review of Laboratory Methods

Sehrawat K S[1*], Patnaik R[2], Naik S[3], Chauhan P[4], Saha A[5]

[1]PhD Scholar, Department of Clinical Research, Galgotias University, Greater Noida, Uttar Pradesh (India), [2]Professor & Dean, Department of Clinical Research, School of Biosciences & Biomedical Engineering, Galgotias University, Greater Noida, [3]Senior Resident, Department of Biochemistry, LHMC & Associated Hospitals, New Delhi, [4]Associate Professor, Department of Biochemistry, LHMC & Associated Hospitals, New Delhi, [5]Professor, Division of Pediatric Nephrology, Department of Pediatrics, Kalawati Saran Children's Hospital, New Delhi.
*kaptansinghsehrawat@yahoo.co.in

ABSTRACT

Tacrolimus is an immunosuppressive agent prescribed in various medical conditions like organ transplantation, malignancies, autoimmune diseases, and for the treatment of Nephrotic Syndrome. It has a narrow therapeutic index and even at a low trough level (4-6 ng/mL) has been found to be linked with nephrotoxicity. Therapeutic drug monitoring of immunosuppressive drugs is required because of their varied metabolism, absorption, and drug interactions. The pharmacokinetics data of Tacrolimus is also very limited for the treatment of steroid resistant nephrotic syndrome in children. There are various laboratory detection methods which have an important role in treatment outcomes. Aim of this study is to review various laboratory methods in terms of functional feasibility, cost-effectiveness, and turnaround time (TAT) for therapeutic drug monitoring of tacrolimus. Published data of various laboratory detection methods have been evaluated in this study and found that LC-MS and Immunoassay are two important techniques that are applied for the therapeutic monitoring of Tacrolimus. The LC-MS is a gold standard method that requires a high degree of technical competence and extensive training to perform therapeutic drug testing. Turbidimetric immunoassays can also be an alternative to LC-MS in resource-constraint laboratory facilities.

Keywords: Tacrolimus; Steroid Resistant; Nephrotic Syndrome; LC-MS; PETIA; Pediatrics; QMS Tacrolimus Immunoassay

Introduction: Tacrolimus is an important antibiotic of fungal origin, *Streptomyces tsukubaensis* with a potent immunosuppressive function. It is used in multiple clinical conditions including organ transplantation, autoimmune diseases, malignancies, and for treatment of Nephrotic Syndrome [1] It has a very narrow therapeutic index and even at a low trough level (4-6 ng/mL) has been linked to nephrotoxicity [2]. It is a calcineurin inhibitor that inhibits the production of IL-2 and discourages the proliferation of T cells [3, 4]. It is metabolized in the liver, mainly via CYP3A [5]. Common interactions of tacrolimus are with grapefruit, antimicrobials, and anti-fungal which increase the levels. It has high inter-individual and intra-individual pharmacokinetic variability. The pharmacokinetics data of tacrolimus and its correlation with therapeutic efficacy is very limited in the Indian population particularly in children which requires regular therapeutic

monitoring of the drug. It is also important for clinical laboratories to evaluate laboratory monitoring methods which have a crucial role in treatment outcomes. The purpose of this review is to discuss various laboratory methods in terms of their advantages and disadvantages, functional feasibility, cost-effectiveness, and required turnaround time (TAT) for therapeutic drug monitoring of tacrolimus.

Significance of Therapeutic Drug Monitoring of Tacrolimus: A Trough level of tacrolimus is essential in patients to prevent rejection of kidney, heart, or liver transplants [6, 7]. It is useful for determining adequate therapeutic concentration and also to avoid the toxicity of a drug. The management of organ transplant rejection and graft vs. host disease (GVHD) remains a challenging task for the clinician. Tacrolimus toxicity is mostly seen in children when plasma levels exceed 15 to 20 ng/mL which may include life-threatening complications like hyperkalemia, insulin-dependent diabetes mellitus, reversible left ventricular hypertrophy, cardiomyopathy, and encephalopathy[7]. Therefore, proper compliance with immunosuppressive therapy is of utmost importance for long-term survival. Therapeutic drug monitoring of immunosuppressive drugs is essential because of diverse metabolism, absorption, and drug interactions. Timely monitoring of drug levels is also important to improve efficacy and reduce the toxicity of individual drug dosage.

Management of steroid-resistant nephrotic syndrome (SRNS) is always challenging for clinicians. There is a dearth of pharmacokinetic data of tacrolimus regarding nephrotic syndrome in children. Gut edema, heavy proteinuria, hypercholesterolemia, hypoalbuminemia, and hypertriglyceridemia are distinctive features of nephrotic syndrome thus the pharmacokinetic data of organ transplant studies may not be appropriate to children with nephrotic syndrome. Hypoalbuminemia may lead to reduced protein binding, whereas gut edema can lead to uneven absorption of the drug which may also have an altered volume of drug distribution or clearance. Further, renal transplant data also indicates a narrow therapeutic index of a drug, significant inter-individual inconsistency in tacrolimus trough concentration (C0), and inter-dose area under the curve (AUC0–12 h). However, the Tacrolimus has gained clinical acceptance in the management of steroid-resistant nephrotic syndrome (SRNS) in children nevertheless therapeutic range is still extrapolated from pediatric renal transplant recipients due to the limited availability of pharmacokinetic data in SRNS patients.

Laboratory Methods: Various laboratory methods are being applied in laboratories for therapeutic drug monitoring like Liquid Chromatography-Mass Spectrometry (LC-MS/MS), Gas Chromatography-Mass Spectrometry (GC-MS), High-Performance Liquid Chromatography (HPLC), Particle Enhanced Turbidimetric Immunoassay (PETIA), and Dried-blood-spot analysis etc.

Particle Enhanced Turbidimetric Immunoassay (PETIA): The QMS Tacrolimus Immunoassay is a homogeneous particle-enhanced turbidimetric immunoassay. The assay is

based on competition between the drug in the sample and the drug-coated onto a microparticle for antibody binding sites of the tacrolimus antibody reagent. The tacrolimus-coated microparticle reagent is rapidly agglutinated in the presence of the anti-tacrolimus antibody reagent and the absence of any competing drug in the sample. The rate of absorbance change is measured photometrically at 700 nm. When a sample containing tacrolimus is added, the agglutination reaction is partially inhibited, slowing down the rate of absorbance change. A concentration-dependent classic agglutination inhibition curve can be obtained with the maximum rate of agglutination at the lowest tacrolimus concentration and the lowest agglutination rate at the highest tacrolimus concentration. [8]

Liquid Chromatography-Mass Spectrometry (LC-MS): Tacrolimus (TAC) has a narrow therapeutic index and a high inter-individual and intra-individual pharmacokinetic variability, which necessitates therapeutic drug monitoring to individualize dosage. Mass spectrometers operate by converting the analyte molecules to a charged (ionized) state, with subsequent analysis of the ions and any fragment ions that are produced during the process, based on their mass to charge ratio (*m/z*). [10] Liquid chromatography-mass spectrometry (LC-MS) is now a commonly used technique with the development of electrospray ionization (ESI) providing a simple and robust interface [9]. LC-MS is mostly preferred in labs due to its high specificity and reduced run time. This technique uses molecular fragmentation for the separation of particles. Single quadrupoles, triple quadrupoles, and quadrupole ion-trap instruments are the most used mass analyzers in routine laboratories, in the fields of forensic toxicology and therapeutic drug monitoring [11]. Lower drug concentrations can also be quantified. It further helped in the quantification of lower drug concentrations in the blood samples. The advantages of HPLC-mass spectrometry are high sensitivity, specificity, small sample requirements, minimal sample preparation, rapid throughput, and simultaneous measurement [12]. The application of immunoassay methods may lead to an over-estimation of blood trough levels and under-dosage of the drug. Overestimation of the concentration using immunoassay methods occurs because of the cross-reaction with metabolites. The occurrence of such overestimation indicates the general need for more precise methods for drug monitoring. The high selectivity of LC/MS/MS methods prevents an overestimation of the concentration of immunosuppressive agents in patient samples [13]. The disadvantage of this method is that it required high upfront costs and full validation for use. However, to perform this LC-MS testing; a high degree of technical ability and extensive training is required.

High-Pressure Liquid Chromatography (HPLC): It is the sensitive and specific method used for measuring the tacrolimus. The stationary phase used is usually a C18 column and the mobile phase is methanol with formic acid. The principle of separation is adsorption. When a mixture of components is introduced into an HPLC column, with high pressure they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards adsorbent generally travels slowly. The component which has less affinity will travel faster finally the components get separated. They were mostly combined with techniques like ELISA

and mass spectrometry for the analysis of tacrolimus. The disadvantages of HPLC-MS were the high cost of equipment and the availability of suitably skilled scientific staff. The advantages of HPLC-mass spectrometry were high sensitivity, specificity, small sample requirements, minimal sample preparation, rapid throughput, and simultaneous measurement [14].

Gas Chromatography-Mass Spectrometry (GC-MS): This is a method that uses very high temperature for causing sample vaporization. Vaporized fractions are then passed through the electric field where they get separated based on their molecular weight. The pattern of separation is unique for each drug, therefore establishes a fingerprint for identification [15]. GC MS has limited use as it is preferable only for volatile substances. GC MS is not the preferred method these days.

Radio Immuno Assay (RIA): It generally, uses radioactivity for the detection of the presence of the analyte. In RIA the sample is incubated with an antibody and a radiolabeled drug (mostly used radio labeled substance I125). The amount of radioactivity measured is compared to the radioactivity present in the known standards which are included in each run and results are quantitated. Mostly used of determination of drugs of abuse. The advantages of RIA were high specificity and sensitivity. The side effects mostly included radiation hazards, the use of radio labeled reagents, the requirement of specially trained persons. Labs also require a special license to handle radioactive material. Further a special arrangement for storage, and waste disposal of radioactive materials is also a big challenge [16].

Particle Enhanced Turbidimetric-Inhibition Immunoassay (PETINIA): It is also an immune turbidimetric method. It mostly uses the creation of light scattering particles to measure drug levels**.** The free drug in the sample competes for the antibody fragment, thereby decreasing the rate of particle aggregation. The rate of aggregation is inversely proportional to the concentration of a drug in the sample. It's a developing technique and not much literature is available.

Cloned Enzyme Donor Immunoassay (CEDIA) : Competitive homogenous enzyme immunoassay. It uses a genetically formulated enzyme βgalactosidase. This assay has two component fragments of an enzyme: enzyme acceptor and an enzyme donor which are generally inactive, but in solution, they become activated and reassemble. As a single subunit, they can react with the substrate. Drug bound to the enzyme donor competes with the drug or with metabolite in the sample for antibody binding site. If the drug bound to the enzyme donor binds to the antibody, it is prevented from reassembling with the enzyme acceptor and activating the enzyme. If the drug is present the unbound enzyme donor reassembles with the enzyme acceptor and reacts with the substrate to produce a change of absorbance. Linearity generally ranges from 0-30 ng/ml. It is well correlated with the gold standards LCMS (r = 0.964) and MEIA (r =0.874) [17]. The reportable range varies from 2-30 ng/ml. Moreover follows 2-point calibration throughout standard curve assay.

Chemiluminescent Microparticle Enzyme Immunoassay (CMIA): Before the initiation of the procedure, a manual pretreatment step is performed in which the whole blood sample is extracted with a precipitation reagent and centrifuged. The supernatant is transferred into a Transplant Pretreatment Tube, which is placed onto the System. Sample, assay diluent, and anti-tacrolimus coated paramagnetic microparticles are combined to create a reaction mixture. Tacrolimus present in the sample binds to the anti-tacrolimus coated microparticles. After a delay, tacrolimus acridinium-labeled conjugate is added to the reaction mixture. The tacrolimus on the acridinium-labeled conjugate competes for the available binding sites on the microparticles. Following incubation, the microparticles are washed and pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). An indirect relationship exists between the amount of tacrolimus in the sample and the RLUs detected by the system optics. A study by Marubashi, *et al* had compared the method with MEIA and found that the correlation between the two methods was highly significant ($r = 0.941$). They found that CMIA was much superior to MEIA in detecting the low levels [18].

Dried Blood Spot (DBS): In this method sampling using a finger prick is an emerging alternative to venous sampling. Important advantages of DBS sampling include the less amount of blood is needed, the possibility of home-based sampling after which the sample can be sent to the laboratory, easier sampling at desirable sampling times (eg, trough concentrations), and the quick results being available to the patient before the next outpatient visit[19]. This process of TDM with DBS sampling has recently been demonstrated to be cost-effective, with lifelong concentration monitoring [20] There has been an increase in the development of DBS assays for TDM for a wide range of drug therapies, including immunosuppressants [21, 22]. The challenge is to increase the applicability of DBS sampling as adequate clinical validation is required. Second, the acceptability of DBS sampling needs to be evaluated to assess the possible bottlenecks for implementation at an early stage. Only then can widespread home-based sampling for TDM can be implemented.

Discussion: The adjustment of dose for tacrolimus based on daily therapeutic drug monitoring (TDM) is important to prevent rejection and its severe adverse reactions like central neurotoxicity and nephrotoxicity. The factors like hematocrit, plasma proteins as well as drug concentration are known to affect the distribution of tacrolimus between whole blood and plasma [23, 24]. Tacrolimus is known to be metabolized by CYP3A into at least 8 metabolites through demethylation and hydroxylation [25]. Therefore frequent monitoring of the drug is recommended. LC-MS/MS is used as the primary chromatographic detection method across the world due to its high specificity and sensitivity [26]. Detection of tacrolimus by immunoassay is another preferred method nowadays. These methods mostly include the use of anti-tacrolimus antibodies conjugated with specific antigens. Antibodies used in immunoassay are well known to cause cross-reactivity with a variety of metabolites [27]. Advances in immunoassay measurement involve automated specimen pretreatment, enhanced reagent stabilities to lower potential matrix effect, and new anti-Tac antibodies that provide more sensitivity and affinity to the target drug.

Immunoassay continues to be used in many laboratories across the country because of its ease of use and lower costs associated with services. Many laboratories find this option appealing because it does not involve a high level of technical skill from staff; the equipment can be leased; and the manufacturer often provides training, support, and maintenance for these systems. A study held in Japan had mentioned that the Microparticle enzyme immunoassay was the most widely used method for more than 20 years. After that, another new technique was introduced as chemiluminescent enzyme immunoassay (CLIA and an affinity column-mediated immunoassay had been introduced and used in Japan. These 2 immunoassay methods were based on anti-tacrolimus antibodies, which had different properties in the cross-reaction with tacrolimus metabolites. Tacrolimus concentrations were measured in the peripheral blood of 102 patients using MEIA, CLIA, ACMIA, and LC-MS/MS. Additional blood samples of 54 patients, who also underwent liver transplantation at Kyoto University Hospital, were analyzed using the newly developed FIA-MS/MS and LC-MS/MS. CLIA had shown the highest accuracy among all the 3 assays [28]

Another study by Marubashi et al also mentioned that CLIA had a highly significant association with the results of the gold standard LC-MS (r=0.964)[18]. With the added influence of pharmacokinetic and pharmacodynamic factors affecting drug therapy, continuous monitoring of drug concentration is recommended. Since the physicians need to maintain the Tacrolimus levels between 3–7 ng/ml, methodologies capable of quantifying up to 1ng/ml need to be developed. Techniques like LC-MS, CLIA, ACMIA, and newer techniques Like PETIA and CEDIA have made rapid assays possible.

Although there has been literature suggesting LC-MS as the gold standard assay due to its high specificity it has a major disadvantage of a high-ended and costly setup, moreover requires extremely trained professionals. Ultimately labs now prefer immunoassays as they are more cost-effective and do not require specialized training. Although the major disadvantage the immunoassays exhibit is cross-reactivity with different metabolites, with advances in technology newer methods like PETIA, CEDIA, and Dried blood spot analysis have been implemented. Studies have exhibited that these techniques significantly correlated with the gold standard techniques and they were found to be cost-effective. CEDIA correlated well with LC-MS (r= 0.924) and could detect TAC concentration between1-30ng/ml. It further followed 2 point calibration throughout standard curve assay. On the other hand, PETIA is emerging as the most cost-effective procedure nowadays. Correlation studies also established a highly significant association between test specimens with PETIA to that of LC-MS (r = 0.972) and the Abbott Architect assay (r=0.937). PETIA can detect as low as 1ng/ml with linearity of 0-30ng/ml. It also follows 6 point calibration which makes this technique highly sensitive. Dried spot analysis using finger prick is usually a less invasive, painless method for clinical analysis [20] but this technique needs proper validation for implementation.

Conclusion: LC-MS and Immunoassay are two important techniques that are used for the therapeutic monitoring of Tacrolimus. The LC-MS is a gold standard method which requires high costs and full validation for use. A high degree of technical ability and extensive training is required to perform this type of testing. Turbidimetric immunoassays can be considered a better alternative to LC-MS in terms of cost and turnaround time (TAT), especially in resource-constraint laboratory facilities.

References:

1. Thölking G, Gerth HU, Schuette-Nuetgen K, Reuter S. Influence of tacrolimus metabolism rate on renal function after solid organ transplantation. World J Transplant. 2017 Feb 24; 7(1):26-33.

2. Starzl T, Todo S, Fung J, et al. FK506 for human liver, kidney and pancreas transplantation. Lancet1989; 2:1000–4.

3. McCauly J, Fung J, Jain A, et al. The effects of FK506 on renal function after liver transplantation. Transplant Proc 1990; 22:17–20.

4. Tamura K, Kobayashi M, Hashimoto K, et al. A highly sensitive method to assay FK-506 levels inplasma. Transplant Proc 1987;19: 23–9.

5. Elens L, Capron A, van Schaik RH, De Meyer M, De Pauw L, Eddour DC, Latinne D, Wallemacq P, Mourad M, Haufroid V. Impact of CYP3A4*22 allele on tacrolimus pharmacokinetics in early period after renal transplantation: toward updated genotype-based dosage guidelines. Ther Drug Monit. 2013 Oct; 35(5): 608-16.

6. Port FK, Dykstra DM, Merion RM, Wolfe RA. Trends and results for organ donation and transplantation in the United States, 2004. Am J Transplant. 2005;5(4 Pt 2):843–849. 6. Gounden V, Soldin SJ. Tacrolimus measurement: building a better immunoassay. Clin Chem. 2014;60(4):575–576.

7. Scott LJ, McKeage K, Keam SJ, et al. FK506: a further update of its use in the management of organ transplantation. Drugs. 2003;63:1247-1297

8. Christians U, Pokaiyavanichkul T, Chan L, Tacrolimus, Applied Pharmacokinetics and Pharmacodynamics: Principals of Therapeutic Drug Monitoring. 4th Edition. Lippincott Williams & Wilkins, Philadelphia, PA 2005: 529-562.

9. Ho CS, Lam CWK, Chan MHM, Cheung RCK, Law LK, Lit LCW, et al. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin Biochem Rev. 2003;24:3–12.

10. Gallardo E, Barroso M, Queiroz JA. LC-MS: a powerful tool in workplace drug testing. Drug Test Anal. 2009 Mar;1(3):109-15

11. Jansen R, Lachatre G, Marquet P. LC-MS/MS systematic toxicological analysis: comparison of MS/MS spectra obtained with different instruments and settings. Clin Biochem. 2005;38:362–72

12. Yang Z, Wang S. Recent development in application of high performance liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressants. J Immunol Methods. 2008;336:98–103

13. Agrawal YP, Cid M, Westgard S, Parker TS, Jaikaran R, Levine DM. Transplant patient classification and tacrolimus assays: more evidence of the need for assay standardization. Ther Drug Monit. 2014;36(6):706–709.

14. Taylor PJ. Therapeutic drug monitoring of immunosuppressant drugs by high-performance liquid chromatography-mass spectrometry. Ther Drug Monit. 2004 Apr;26(2):215-9.

15. Kapur BM. Drug-testing methods and clinical interpretations of test results. Bull Narc. 1993;45(2):115-54.

16. Mould G.P., Aherne G.W., Morris B.A. *et al*. Radioimmunoassay of drugs and its clinical application. *European Journal of Drug Metabolism and Pharmacokinetics* 2, 171–184 (1977)

17. Liang SL, Breaud A, Dunn W, Clarke W. Comparison of the CEDIA and MEIA assays for measurement of tacrolimus in organ transplant recipients. Clin Chim Acta. 2008 Oct;396(1-2):1-6.

18. Marubashi S, Nagano H, Kobayashi S, Eguchi H, Takeda Y, Tanemura M, Umeshita K, Monden M, Doki Y, Mori M. Evaluation of a new immunoassay for therapeutic drug monitoring of tacrolimus in adult liver transplant recipients. J Clin Pharmacol. 2010 Jun;50(6):705-9.

19. Demirev P.A. Dried blood spots: Analysis and applications. Anal. Chem. 2013;85:779–789.

20. Zakaria R., Allen K.J., Koplin J.J., Roche P., Greaves R.F. Advantages and Challenges of Dried Blood Spot Analysis by Mass Spectrometry Across the Total Testing Process. EJIFCC. 2016;27:288–317.

21. Hinchliffe E, Adaway JE, Keevil BG. Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2012; 883–884: 102–107.

22. Koop DR, Bleyle LA, Munar M, Cherala G, Al-Uzri A. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2013; 926: 54–61

23. Jusko WJ, Thompson AW, Fung J, et al. Consensus document; therapeutic monitoring of tacrolimus(FK-506). The Drug Monit 1995;17:606-614

24. Physicians' Desk Reference, 58th ed. Thomson PDR at Montvale, NJ. 2004; PROGRAF®: 1323-1327

25. Christians U, Pokaiyavanichkul T, Chan L, Tacrolimus, Applied Pharmacokinetics and Pharmacodynamics: Principals of Therapeutic Drug Monitoring. 4th Edition. Lippincott Williams & Wilkins, Philadelphia, PA 2005: 529-562.

26. Polledri E, Mercadante R, Ferraris Fusarini C, Maiavacca R, Fustinoni S. Immunosuppressive drugs in whole blood: validation of a commercially available liquid chromatography/tandem mass spectrometry kit and comparison with immunochemical assays. Rapid Commun Mass Spectrom. 2017 Jul 15;31(13):1111-1120.

27. Bazin C, Guinedor A, Barau C, et al. Evaluation of the ARCHITECT tacrolimus assay in kidney, liver, and heart transplant recipients. J Pharm Biomed Anal. 2010;53(4):997–1002.

28. Hashi S, Masuda S, Kikuchi M, Uesugi M, Yano I, Omura T, Yonezawa A, Fujimoto Y, Ogawa K, Kaido T, Uemoto S, Matsubara K. Assessment of four methodologies (microparticle enzyme immunoassay, chemiluminescent enzyme immunoassay, affinity column-mediated immunoassay, and flow injection assay-tandem mass spectrometry) for measuring tacrolimus blood concentration in Japanese liver transplant recipients. Transplant Proc. 2014 Apr; 46(3):758-60.