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MICROSAMPLING AND CONVENTIONAL SAMPLING TECHNIQUES FOR QUANTIFICATION OF TACROLIMUS IN BLOOD SAMPLES: A SYSTEMATIC REVIEW

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ABSTRACT

Tacrolimus is immunosuppressive drugs that have a narrow therapeutic range, and a large inter-patient variability in their pharmacokinetics. Serial monitoring of tacrolimus after transplantation is important to ensure the concentration in biological fluids is maintained at the therapeutic range. Blood sampling for therapeutic drug monitoring of tacrolimus is mostly by venipuncture, making it inconvenient and invasive due to repeated sampling. Thus, microsampling has been adopted as an alternative for sample collection as it is less invasive and more convenient for patients. This article aims to review validated bioanalysis methods for tacrolimus to compare the methods using venous sample and microsampling methods. Related studies about tacrolimus analysis method in blood samples were screened from several databases and summarized with PRISMA flow diagram. A total of 12 studies were considered eligible and reviewed in this article. This systematic review provides a narrative of sample quantification of tacrolimus that collected by venipuncture and microsampling method, its bioanalytical method employed to perform the analysis, and clinical validation or application to patients with organ transplants. Studies with microsampling methods have been validated and applied to the patients. Some studies that compared both methods concluded there is no significant difference between tacrolimus concentrations by venipuncture or microsampling methods. Therefore, microsampling can be considered an option for future development of sampling method in tacrolimus routine monitoring.

KEYWORDS *Tacrolimus, Microsampling, Validation,Therapeutic Drug Monitoring*

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INTRODUCTION

Tacrolimus is an immunosuppressive drug that is used after transplantation to prevent organ rejection or graft-versus-host disease. The drugs have a narrow therapeutic range and a large inter-patient variability in their pharmacokinetics (Francke et al., 2022). Therapeutic drug monitoring (TDM) and dose individualization for tacrolimus is essential. Inaccuracies can cause overdosing which has many harmful effects or under-dosing which can lead to allograft rejection (McShane et al., 2016).

Several analytical methods for tacrolimus TDM have been developed for the determination of immunosuppressive drugs such as immunoassay and chromatography. Immunoassays such as microparticle enzyme immunoassay, affinity column-mediated immunoassay, and chemiluminescence microparticle immunoassay analytical methods for the determination of immunosuppressants have been described (Hashi et al., 2014). Most clinical laboratories use LC-MS/MS methods as a gold standard for the TDM of immunosuppressants (Mei et al., 2018). Using MS/MS as a detector allows high sensitivity and the achievement of a very low limit of detection and quantification and high specificity by detecting the signature mass-to-charge ratio (m/z), the drug and its similar metabolites can be discriminated (Zhang & Zhang, 2018).

Tacrolimus is routinely measured in venous whole blood samples collected by venipuncture (Hinchliffe et al., 2012). Recently, microsampling approaches have been increasingly considered useful alternatives to conventional venous sampling because of their minimal invasive manner. With the improvements in molecular detection, the amount of starting sample quantity needed has significantly reduced in some diagnostic procedures (Lei & Prow, 2019). Microsampling techniques have been developed for pharmacokinetics study or therapeutic drug monitoring such as antibiotics (Moorthy et al., 2020; Vu et al., 2014), antipsychotics (Bernardo et al., 2022; Jacobs et al., 2021), and anticancer drugs (Harahap et al., 2021; Maggadani et al., 2021; Radovanovic et al., 2022).

In this article, the author conducted a systematic review of bioanalysis of tacrolimus in human blood samples by conventional or microsampling techniques to evaluate the advantages and limitations of the methods. It gives a comprehensive comparison of validated bioanalytical methods for tacrolimus, thereby offering valuable insights for optimizing tacrolimus monitoring in clinical and research settings. In this article, the author systematically selected records on this subject and compared the different sampling techniques and the bioanalytical method to analyze tacrolimus in human whole blood samples.

RESEARCH METHOD

Study protocol

The Preferred Reporting Items for Systematical Reviews and Meta-Analysis (PRISMA) statement is used as a guideline to conduct the literature search (Page et al., 2021). For the search strategy, the following descriptors were employed:

tacrolimus, dried blood spot (DBS), volumetric absorption microsampling (VAMS), and LC-MS/MS using the Boolean operator. The search was conducted in ScienceDirect, PubMed/Medline, and Google Scholar. The potential studies were screened according to the inclusion and exclusion criteria.

Inclusion and exclusion criteria

The inclusion criteria for this article review were: (1) Experimental studies written in English, (2) Studies that explained sampling techniques and analytical methods of tacrolimus in human whole blood samples. The exclusion criteria were (1) studies written in other languages besides English, (2) non-related studies, (3) duplicate publications, (4) articles not available as full text, and (5) full validation and clinical application not written.

Selection of studies and data collection

All search results were collected and screened. The identified studies are assessed based on the title and abstract without using any specific data extraction form. Then, the eligible articles were fully reviewed according to inclusion and exclusion criteria. The data are gathered independently according to study design, sample matrix, and bioanalytical method development.

RESULT AND DISCUSSION

A total of 624 potentially relevant studies were identified through database searching. The screening was conducted based on the inclusion and exclusion criteria that had been established, resulting in 12 articles considered eligible for this review. Other articles were rejected because they used another method, and didn't have full validation, clinical validation, or clinical application. The PRISMA flow diagram of the article screening process can be seen in Figure 1.

Tacrolimus

Tacrolimus ($C_{44}H_{69}NO_{12}$) also known as FK-506 is a 23-membered macrolide ring produced by *Streptomyces tsukubaensis*, has a molecular weight of 803.5 g/mol and is highly lipophilic (Log P = 3.3). Tacrolimus, or by its trade names Prograf and Advagraf (both manufactured by Astellas Pharma, Inc) was discovered in 1984 by Fujisawa Pharmaceutical Co Ltd (Kalt, 2017). Tacrolimus is formulated as a prolonged-release capsule that suppresses interleukin-2 production associated with T-cell activation thus inhibiting differentiation and proliferation of cytotoxic T-cells (Fung, 2004).

In blood, tacrolimus was found to be mainly associated with erythrocytes, followed by diluted plasma proteins and lymphocytes. In plasma, tacrolimus was found to mainly be associated with the soluble protein fraction, high-density lipoproteins, low-density lipoproteins, and very low-density lipoproteins (VLDL) (Zahir et al., 2001). Therefore, whole blood, and not plasma or serum, is the chosen matrix for the determination of tacrolimus concentrations (de Loor et al., 2021). However, there was a study that used plasma and lymphocytes to determine tacrolimus concentration (Masri et al., 2013; Romano et al., 2018). Tacrolimus

provided better efficacy than cyclosporin in terms of patient and graft survival, treatment failure, and the incidence of acute and corticosteroid-resistant rejection episodes (Scott et al., 2003).

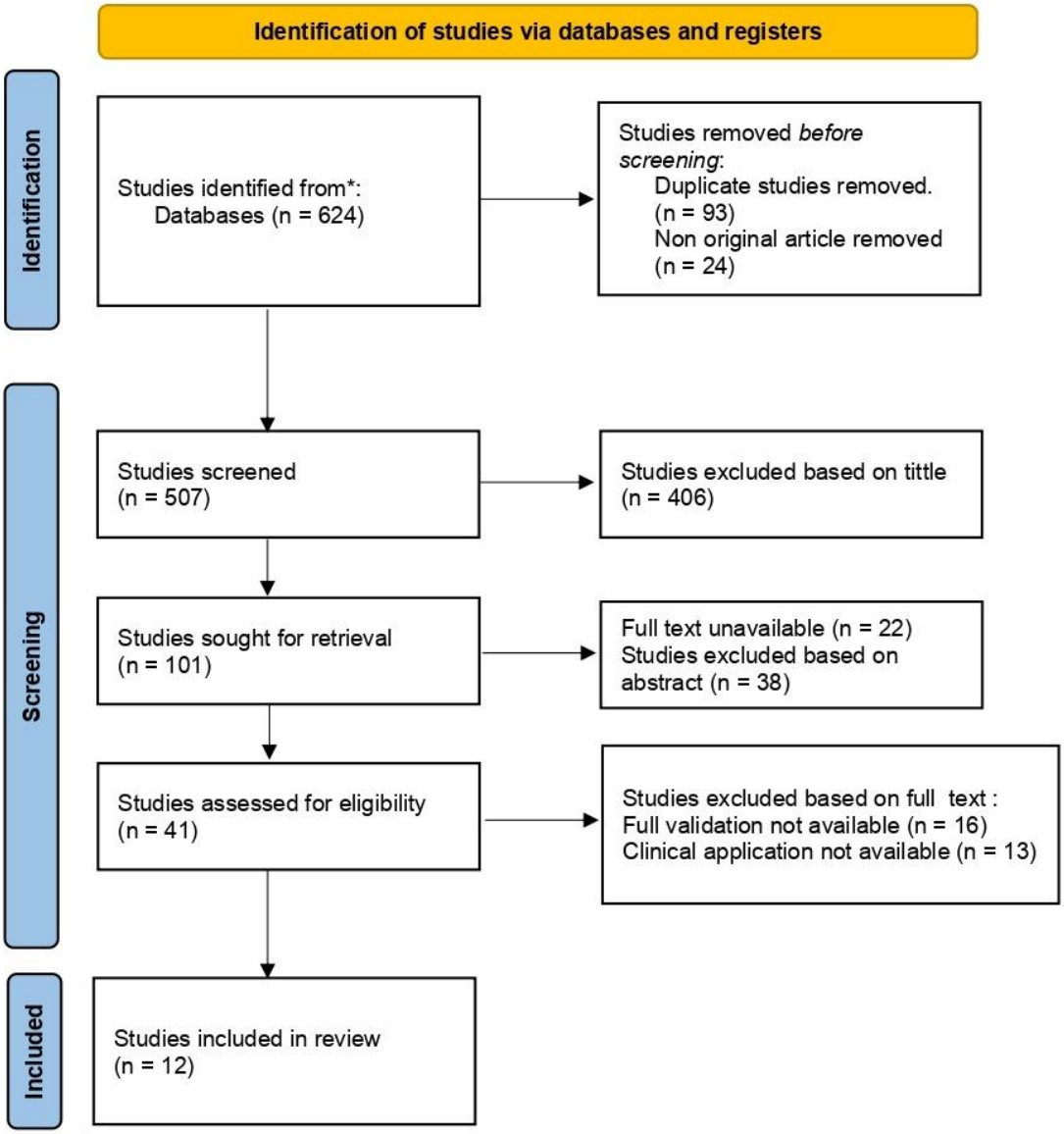

Figure 1. PRISMA flow diagram of the study search process

Frequent and careful determinations of tacrolimus in whole blood trough levels are necessary to prevent the risk of adverse reactions, which largely consist of nephrotoxic and neurotoxic effects, as well as new-onset diabetes mellitus, after transplantation (Kalt, 2017). This review covered 8 studies that analyze tacrolimus, including studies combining other immunosuppressive drugs (Cyclosporine A, Mycophenolic acid, sirolimus, everolimus), its metabolites, and creatinine.

Sampling Method

The collection of blood samples by phlebotomy is the most common type of biological specimen collection (Ialongo & Bernardini, 2016). The blood is usually obtained from the median cubital vein in the upper arm, near the radial cutaneous vein. A tourniquet is applied to the arm and then the area will be cleansed with alcohol. Patients are asked to close their hands, venipuncture is performed with a suitable needle, and once the blood flow begins, patients will be requested to open their hands, the tourniquet released, and the blood will be collected using a tube. This method can be applied for plasma and serum sampling, in which the tube for plasma collection contains an anticoagulant and the tube for serum does not contain an anticoagulant. This method is relatively invasive, and in clinical trials may lead to a low recruitment rate, especially in paediatric patients. The quality of the phlebotomy will also directly affect the quality of the sample and the result of the analysis (Lima-Oliveira et al., 2015). In this review, 6 studies have been described to determine tacrolimus in whole blood including 3 studies that compare with microsampling.

DBS as microsampling technique has been introduced more into clinical practice to facilitate TDM. With this method, a drop of capillary blood, taken from the finger or heel pricks in young children and neonates, is collected on a filter paper and dried for about 2 to 3 hours. After the blood has been dried, the spot is punched and can be directly analyzed using suitable analytical techniques or stored and transported to an analytical laboratory (Capiau et al., 2019). The advantages of DBS sampling are the minimally invasive, small volume of blood needed, easy procedure, convenient storage, and transport. The patients can perform the finger prick at home so there is no need for hospital visits (Wilhelm et al., 2014). In this review, 5 studies have been described to use DBS for tacrolimus analysis in human blood samples.

VAMS is a microsampling method with the device to collect peripheral blood. The device includes a plastic handle and a globous tip, made from a proprietary hydrophilic polymer, with a diameter of about 4 mm, and can absorb a fixed volume of sample into its pores (10 µL, 20 µL, or 30 µL). The VAMS device has the same advantage as DBS, but VAMS has advantages over DBS, related to sampling volume accuracy, pre-treatment, automation, and hematocrit effect that can lead to inhomogeneity due to the presence of blood cells that make different concentrations of the analytes and matrix compounds/interferents (Protti et al., 2019). In this review, 5 studies have been described to use VAMS for tacrolimus analysis in human blood samples.

Tacrolimus has narrow therapeutic concentration and large variability, so the drug level in patients who receive this drug should be monitored frequently even after the patient has been discharged from the hospital. Because of this, sampling for monitoring will be difficult or delayed because the patient must come to the hospital. With the microsampling method, the patient could puncture a finger with a lancet, place the blood on filter paper or a VAMS device, and mail the sample to the laboratory. Since the patient is not encumbered by frequent trips to a blood drawing facility more samples could be collected for more frequent TDM (McShane et al., 2016). To ensure that drug concentrations are not biased due to

microsampling device effects and/or absorption of the drug into the microsampling device, clinical validation should be carried out.

In DBS, hematocrit, both low and high levels, was reported to have a significant influence on the immunosuppressant results. Higher hematocrit samples are more viscous due to the increase of red blood cells, and a viscous sample is less permeable on the paper. Therefore, different size spots are formed depending on hematocrit (McShane et al., 2016)

Francke et al. study observed, that for most of the samples, the drug concentrations measured using DBS were slightly different from drug concentrations measured in whole blood. These results indicated a small systematic bias regarding the effect of the filter paper. The extraction recovery rate of the blood from the filter paper is not 100% and varied (75-116%), and consequently, lower concentrations can be measured in the DBS samples. This can be solved by correction factor and hematocrit effect in clinical validation. No effect of the sample location was observed as concentrations of whole blood samples obtained by a finger prick were similar to concentrations obtained by venous blood sampling (Francke et al., 2022).

Table 1. Summary of LC MSMS method for tacrolimus analysis

Analyte	Matrix	Mobile phase, flow rate	IS	Preparation	TAC LLOQ (ng/mL)	Run time (min)	TAC Recovery (%)	Ref.
TAC, diltiazem	WB	Isocratic: methanol–water (containing 2mM ammonium acetate) (95:5, v/v), 0.2 mL/min	ASC	LLE	0.50	2	58.3 – 62.6	(Li et al., 2008)
TAC and its metabolites	WB	Gradient : MQwater and acetonitrile. 5 mL/min	13CD4-TAC	Precipitation	0.035	6	93 – 110	(de Loor et al., 2021)
TAC	WB	Gradient: A (2 mmol/ammonium acetate and 0.1% formic acid in water) and B (methanol), 0,5 mL/min	ASC	Precipitation	0.37	3.5	86.1 - 114.6	(Yu et al., 2022)

Analyte	Matrix	Mobile phase, flow rate	IS	Preparation	TAC LLOQ (ng/mL)	Run time (min)	TAC Recovery (%)	Ref.
CYA, TAC	WB and DBS	Gradient: A (2 mM ammonium acetate and 0.1% formic acid in water) and B (2 mM ammonium acetate and 0.1% formic acid in methanol), 0.5 mL/min	13C,2H4-TAC	WB: precipitation, DBS: precipitation + sonication	DBS: 2.00 WB: 1.00	2	DBS 75 – 116	(Francke et al., 2022)
TAC	WB and DBS	Gradient: A (2 mM ammonium acetate in water, 0.1% formic acid) and B (2 mM ammonium acetate in methanol, 0.1% formic acid), 0.75 - 3 mL/min.	ASC	WB: precipitation, DBS: dilution + SPE	1.00	6.5	VAMS 78 ± 3.5	(Hoogtanders et al., 2007)
TAC, MPA, SIR, EVE and CYA	WB and VAMS	Gradient: A (2 mM ammonium formate and 0.1% formic acid in water) and B (Acetonitrile), 0.5 mL/min	13C,d2-TAC	WB: Precipitation VAMS: Precipitation	0.50	2.5	VAMS 76.5 - 87.0	(Paniagua-González et al., 2020)
TAC	DBS and VAMS	Gradient: A (2 mM ammonium acetate buffer with 0.1% formic acid) and B (2mM ammonium acetate in methanol with 0.1% formic acid), 0.5 mL/min	ASC	DBS: precipitation VAMS: precipitation	1.46	3	DBS 79 - 85.6 VAMS: 100.5-101.5	(Mathew et al., 2022)

Analyte	Matrix	Mobile phase, flow rate	IS	Preparation	TAC LLOQ (ng/mL)	Run time (min)	TAC Recovery (%)	Ref.
TAC, MPA	DBS	Gradient: A (2 mM ammonium acetate and 0.1% formic acid in water) and B (2 mM ammonium acetate and 0.1% formic acid in methanol). 0.75 – 3 mL/min	ASC	SPE	2.50	6.5	76 - 104	(Martial et al., 2017)
TAC, SIR, EVE and CYA	DBS	Gradient: Methanol and 20mM ammonium formate buffer pH 3.5. 0.5 mL/min	13C,2H2 TAC	Precipitation	1.00	3.1	95.2 - 95.4	(Koster et al., 2013)
TAC, MPA	VAMS	Gradient: A (2 mM ammonium acetate and 0.1% formic acid in water) and B (2 mM ammonium acetate and 0.1% formic acid in methanol). 0.30 mL/min	13C,2H4 TAC	Precipitation	0.50	5	96.49 – 105.89	(Wang et al., 2022)
TAC	VAMS	Isocratic: 95% acetonitrile and 5% 10 mM ammonium acetate in water. 0.1- 0.6 mL/min	ASC	Precipitation + salting out	2.25	3.5	81 - 104	(Tron et al., 2021)
TAC	VAMS	Gradient: A (2 mM ammonium acetate and 0.1% formic acid in water) and B (2 mM ammonium acetate and 0.1% formic acid in methanol). 0.45 mL/min	$[^{13}C_1,^{2}H_2]$ TAC	Precipitation	2.00	5	89.1 ± 14.5 %	(Zhao et al., 2024)

Note = WB : whole blood, DBS: dried blood spot, VAMS: Volumetric absorption microsampling, SPE: Solid phase extraction, LLE: Liquid-liquid extraction, MPA: Mycophenolic acid, TAC: tacrolimus, SIR: sirolimus, EVE: everolimus and CYA: cyclosporin A, ASC: Ascomycin

VAMS is the preferred single sampling option for estimation of tacrolimus, to ensure uninterrupted monitoring from home after organ transplantation when limited by long-distance travel. Moreover, most patients prefer a finger prick over venous blood sampling (Francke et al., 2022). Microsampling can replace whole blood sampling for tacrolimus trough concentration monitoring, but VAMS sampling is preferable to DBS sampling regarding sample quality.

Instrumentation and sample preparation

In recent years, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been successfully applied to clinical immunosuppressive drug TDM. To date, all LC-MS/MS analytical procedures were, by necessity, in-house methods that required extensive development and validation, often with the use of calibration standards and quality control materials prepared in-house (Napoli et al., 2010).

Tacrolimus is present in low levels with a therapeutic range of 5–10 ng/mL for 12 hours through whole blood concentrations, so it needs a sensitive instrument to analyze it (Li et al., 2008). Tacrolimus method must have a low LLOQ concentration, so the concentration of sample can be obtain through the sensitive instrument. Using MS/MS as a detector allows high sensitivity and the achievement of a very low limit of detection and quantification. Major impacts of MS include confirmation of immunoassay-positive drug screens, identification of inborn errors of metabolism, analysis of steroid hormones, and improved time required for microbial identifications (Jannetto & Fitzgerald, 2016). All 12 studies in Table 1 used LC-MS/MS with a positive ion source.

LC-MS/MS's superior specificity makes it the presumptive gold standard in immunosuppressant quantitation. It relieves the method from common interferences that plague immunoassays; such as metabolites that have structural resemblance and interfering antibodies. This increased selectivity also allows everolimus and sirolimus to be differentiated (McShane et al., 2016).

A stable isotope-labeled internal standards (SIL-IS) used when performing bioanalysis with mass spectrometry detection. Using SIL-IS, the potential matrix effect should be adequately compensated. Some of the studies used tacrolimus deuterated as an internal standard, but another used its structural analog, ascomycin. The results of method validation with ascomycin, as an internal standard were satisfying enough to consider that ascomycin, was an acceptable internal standard. Ascomycin was eluted almost at the same retention time as tacrolimus and appeared to compensate matrix effect likely because of the similarity in the chemical structures of both compounds. Matrix effect from all studies with ascomycin and deuterated internal standard results meet the acceptance criteria.

Tacrolimus is analyzed alongside other compounds in a mixture, like with internal standard, metabolite, or other immunosuppressant drugs. Gradient mobile

phase composition of solvents changes over time so it can be adjusted according to the polarity of each analyte and give improved resolution between tacrolimus and other analytes. Other reasons were the results had better separation and more accurate quantification, faster separation for highly heterogeneous samples, and reduced effort in method development (Martin, 2019). Method development for isocratic mobile phase might be more challenging for the analysis with multiple analytes.

The basis of mobile phase is water with methanol or acetonitrile. Ammonium acetate was added in the mobile phase to enhance the abundance of the protonated molecular ion or ammonium·molecular ion complex because it is easily fragmented during MS/MS detection (Li et al., 2008). The positive ion mode of ESI tends to acidify the analyte solution. With ammonium acetate, the pH can drop to as low as 4.75 ± 1 in ESI, reflecting the pKa of the acetate buffer. Formic acid provides protons for the LC-MS analysis in positive ionization mode, by producing [M+H]+ ions. Moreover, it has the advantage of not being a strong ion-pair agent and does not suppress MS ionization (Konermann, 2017). Therefore, ammonium acetate was added for the buffering effect within the acidic pH range alone or in combination with 0.1% formic acid.

The sample preparation techniques used in analytical chemistry are protein precipitation, liquid-liquid extraction, and solid-phase extraction. SPE has high selectivity, can be automated but it requires specific cartridges, and can be time-consuming and costly. Tacrolimus is low polar compound that makes it possible to extract from whole blood by conventional liquid–liquid extraction technique. This process included precipitation with acetonitrile and zinc sulfate solution to lyse the cells followed by adding ethyl acetate as extraction solvent. The extraction by ethyl acetate gave the highest extraction recovery than dichloromethane alone or in combination with 1% isoamyl alcohol (Li et al., 2008).

Protein precipitation is used in most preparation methods for tacrolimus because it is more efficient, faster, and less cumbersome than liquid–liquid extraction or solid phase extraction. Methanol or acetonitrile and zinc sulfate is added for protein precipitation. Zinc sulfate in water increased both erythrocyte lysis and the precipitation power, counterbalancing the high proportion of water in the mixture (Tron et al., 2021).

Validation

Validation is performed according to guidelines from the European Medicines Agency (EMA) or Food and Drug Administration (FDA) (European Medicines Agency, 2010; Food and Drug Administration, 2018). All parameters must meet each of the criteria for the method considered valid.

Selectivity is done to verify that the analytical method can differentiate analyte and internal standard from interference or other components in the sample. Carry over testing means to detect and quantify any potential contamination or residual analytes that remain in the analytical system after analyzing a sample with a high concentration. The acceptance criteria are the responses for selectivity of blank sample is less than 20% from LLOQ analyte and less than 5% for internal standards . Meanwhile, carry over should not exceed 20% of LLOQ. (European

Medicines Agency, 2010; Food and Drug Administration, 2018). LLOQ is the lowest concentration value of the analyte that can still be calculated accurately and precisely. The LLOQ value of the sample must be 5 times greater than the blank and not higher than 5% Cmax (European Medicines Agency, 2010; Food and Drug Administration, 2018).

The calibration curve is the range of concentrations needed to analyze a sample according to the response of the instrument. The calibration curve must contain six concentration levels including ULOQ and LLOQ. The calibration curve must be reproducible and continuous. The concentration measured must be within 15% of the actual concentration, with an exception for LLOQ should be within 20%. (European Medicines Agency, 2010; Food and Drug Administration, 2018).

Accuracy describes the closeness of the obtained concentration from the analysis with the actual concentration value, expressed in percentage. Precision describes the repeatability of the analyte measurement and is expressed as a coefficient of variation (CV). Accuracy and precision are analyzed using quality control (QC) samples in four concentrations, that are LLOQ, QCL (three times the LLOQ), QCM (30–50% of the calibration curve range), and QCH (75% of the ULOQ). Accuracy and precision must be done in a minimum of five replicates of within-run and between-run. Accuracy must be within 15% of the actual concentration, except LLOQ 20%. Precision from the value of CV must not exceed 15%, except LLOQ 20% (European Medicines Agency, 2010; Food and Drug Administration, 2018).

Dilution of samples should not affect accuracy and precision. Dilution integrity testing is done by analyzed diluted analyte above the ULOQ with the blank matrix. For the microsampling method, dilution is done when reconstitution with its suitable solvent. The acceptance criteria are that the accuracy and precision value must be within 15% (European Medicines Agency, 2010; Food and Drug Administration, 2018).

Because tacrolimus in this review used LC MSMS, matrix effects must be analyzed to investigate the effect of ion suppression or ion enhancement on the concentration of the analyte. The matrix factor of the analyte and the internal standard is calculated from the peak area of a spiked matrix with the peak area of the analyte in the absence of a matrix. The acceptance requirement is the CV should not exceed 15% (European Medicines Agency, 2010; Food and Drug Administration, 2018).

Stability testing is carried out to ensure that the process of preparation, analysis, and storage conditions of samples does not affect the concentration of the analyte. The acceptance criteria is that the %diff for each concentration level must not exceed 15%. The stability conditions that must be evaluated are stability of the stock solution, working solutions, and internal standard, freeze and thaw of the analyte on the matrix, short-term stability of the analyte at room temperature, long-term stability of the analyte under storage conditions in the freezer, and autosampler stability (European Medicines Agency, 2010; Food and Drug Administration, 2018)

All of the studies have described a full validation of the analytical method according to the EMA and FDA guidelines with satisfactory results. Paniagua-Gonzalez et al. conducted a study to analyze tacrolimus and other

immunosuppressive drug concentrations in whole blood and VAMS. Clinical validation with 56 transplant recipients found that statistically Hct does not affect the difference in measurement of the two methods (Paniagua-González et al., 2020). Mathew et al. compare DBS and VAMS samples for the estimation of tacrolimus and creatinine which are the two most vital parameters in renal transplantation. There was no statistically significant effect for either analyte by a one-way analysis of variance (ANOVA) concerning the hematocrit of DBS and VAMS (Mathew et al., 2022).

All studies have been proven to apply to transplant patients to study drug concentration levels for pharmacokinetic study, therapeutic drug monitoring, or correlation study between methods. The correlation study showed that DBS and VAMS methods were capable of being used as an alternative for whole blood analysis in therapeutic drug monitoring.

Paniagua-Gonzalez et al. study has better conditions, such as using VAMS, deuterated internal standard, short analysis time, and low LLOQ compared to other methods. This study develops and validates a LC–MS/MS method for the simultaneous determination of tacrolimus and other immunosuppressive drugs in whole blood and VAMS, proving no hematocrit effect (from 0.2 to 0.62) at the required concentration ranges for TDM. The range of concentration from 0.5 to 50 ng/mL was enough to determine TDM concentration and lower than most of other studies. The result of stability testing was within acceptance criteria to ensure the sample that patient sent from home without worrying about the degradation of analyte during the shipping process.

CONCLUSIONS

Several bioanalytical methods have been employed to determine tacrolimus concentration in human blood samples through the means of conventional sampling and microsampling. The amount of literature describing microsampling, such as DBS and VAMS has been increasingly emerging lately. There were studies that have compared whole blood and microsampling, with the result that there is no significant difference between tacrolimus concentrations in both methods. VAMS may be a more convenient option for patient sampling and analysis.

Conflict of Interest

The authors declare no conflict of interest

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