Measuring Intracellular Concentrations of Calcineurin Inhibitors: Expert Consensus from the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Expert Panel

Florian Lemaitre, PhD,†‡ Nils T. Vethe, PhD,§ Antonio D'Avolio, PhD,¶ Camille Tron, PhD,*†‡ Ida Robertsen, PhD,‖ Brenda De Winter, PhD,** Amedeo Denicolo, PhD,¶ Birgit C. P. Koch, PhD,** Raman Venkataramanan, PhD,†† Teun Van Gelder, PhD,**‡‡ Mercè Brunet, PhD,§§ Stein Bergan, PhD,§‖ Dennis A. Hesselink, PhD,‡‡ and Pierre Wallemacq, PhD¶¶*

Background: Therapeutic drug monitoring (TDM) of the 2 calcineurin inhibitors (CNIs), tacrolimus (TAC) and cyclosporin A, has resulted in improvements in the management of patients who have undergone solid organ transplantation. As a result of TDM, acute rejection (AR) rates and treatment-related toxicities have been reduced. Irrespective, AR and toxicity still occur in patients who have undergone transplantation, showing blood CNI concentrations within the therapeutic range. Moreover, the AR rate is no longer decreasing. Hence, smarter TDM approaches are necessary. Because CNIs exert their action inside T lymphocytes, intracellular CNIs may be a promising candidate for improving therapeutic outcomes. The intracellular CNI concentration may be more directly related to the drug effect and has been favorably compared with the standard, whole-blood TDM for TAC in liver transplant recipients. However, measuring intracellular CNIs concentrations is not without pitfalls at both the preanalytical and analytical stages, and standardization seems essential in this area. To date, there are no guidelines for the TDM of intracellular CNI concentrations.

Methods: Under the auspices of the International Association of TDM and Clinical Toxicology and its Immunosuppressive Drug committees, a group of leading investigators in this field have shared experiences and have presented preanalytical and analytical recommendations for measuring intracellular CNI concentrations.

Key Words: tacrolimus, cyclosporin A, pharmacokinetics, transplantation, intracellular

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From the *Department of Clinical and Biological Pharmacology and Pharmacovigilance, Pharmacoepidemiology and Drug Information Center, Rennes University Hospital, Rennes, France; †Rennes 1 University, Faculty of Medicine, Laboratory of Experimental and Clinical Pharmacology, Rennes, France; ‡Inserm, CIC-P 1414 Clinical Investigation Center, Rennes, France; §Department of Pharmacology, Oslo University Hospital, Oslo, Norway; ¶Department of Medical Sciences, University of Turin, Laboratory of Clinical Pharmacology and Pharmacogenetics Padiglione Q, Amedeo, di Savoia Hospital, Turin, Italy; ‖Department of Pharmacy, University of Oslo, Oslo, Norway; **Erasmus MC, University Medical Center Rotterdam, Department of Hospital Pharmacy, Rotterdam, The Netherlands; ††School of Pharmacy, University of Pittsburgh, Pittsburgh, PA; ‡‡Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus MC, University Medical Center Rotterdam, Rotterdam Transplant Group, Rotterdam, The Netherlands; §§Pharmacology and Toxicology Laboratory, CDB, IDIBAPS, Hospital Clinic of Barcelona, University of Barcelona, Spain; and ¶¶Clinical Chemistry Department, Cliniques Universitaires St Luc, Université catholique de Louvain, LTAP, Brussels, Belgium.

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Correspondence: Florian Lemaitre, PhD, Laboratoire de Pharmacologie, Hôpital Pontchaillou, CHU de Rennes, 2, rue Henri Le Guilloux, 35033 Rennes cedex, France (e-mail: florian.lemaitre@chu-rennes.fr).

INTRODUCTION

The concentration of immunosuppressive drug (ISD) in solid organ transplant (SOT) patients determines both the overall therapeutic effects and the adverse effects.[1,2] Using drug level measurements in whole blood, the relationships between ISD concentration and therapeutic and adverse effects have previously been highlighted for 2 calcineurin inhibitors (CNIs), tacrolimus (TAC) and cyclosporin A (CsA).[3–6] Therapeutic drug monitoring (TDM) of TAC and CsA whole-blood trough concentrations is usually recommended as a standard practice in the clinical management of SOT recipients.[1,7] Intensive TDM of TAC and CsA has resulted in significant improvements in the clinical outcomes, including decreased acute rejection (AR) rates and lower treatment-related toxicity (in particular, lower nephrotoxic and neurotoxic effects).

However, despite the intensive TDM, patients still experience AR or toxicity, even when whole-blood concentrations are maintained within the therapeutic range.

Moreover, the AR rate during the first year is no longer decreasing, and AR is commonly reported to remain around 8%–15% with the current triple or quadruple ISD therapies. In studies where patients are monitored with protocol biopsies, AR rates up to 30% have been reported during the first year of treatment.[1,8–11] The apparent limitations of the current TDM approaches and the need for further improvement in the management of transplant recipients, indicate a necessity for newer biomarkers of drug efficacy.

Among these newer approaches, assessing the intracellular concentration of CNIs seems to be a promising candidate. Because CNIs exert their action inside the T lymphocytes, measuring their concentration directly in this biological compartment should provide more relevant results. TAC and CsA are also widely distributed into erythrocytes, probably because of their high concentration of FK506-binding protein 1A (FKBP1A/FKBP-12). As an alternative to whole-blood monitoring of CNIs, the concentration of CNIs in peripheral blood mononuclear cells (PBMCs), a cell fraction enriched in lymphocytes, could be measured. However, CNI concentrations in PBMC have been reported to be only weakly (or not well) correlated with whole-blood concentrations. This may explain why the whole-blood concentration of CNIs does not accurately reflect the drug available to exert the pharmacological effect.[12–15] Drug transporters in the membranes of white blood cells may impact the efflux of ISDs from the active site, and therefore, the intralymphocytic concentration of CNIs may differ substantially in individuals with the same whole-blood concentration.[16,17]

The strategy of monitoring intralymphocytic or intra-PBMC concentrations of CNIs has been favorably compared with conventional, blood-based TDM in the case of TAC administered to patients undergoing liver transplantation.[18] TAC concentrations in PBMC seemed to be tightly correlated with the onset and grade of AR during the first week after liver transplantation, whereas whole-blood TAC concentrations were not.[18,19] Another study has highlighted an association between CsA in T lymphocytes and rejection. The same study has also reported that a decrease in the CsA T-lymphocyte:whole blood C_2 ratio is an early signature of AR in kidney transplant recipients.[20] CNI concentrations in PBMC may also mirror drug concentrations in T cells within lymphoid tissue, where T-cell activation and proliferation occur. Hence, intracellular CNI measurements demonstrate potential as a tool to complement whole-blood TDM of CNIs, with the aim of optimizing transplant patient's care.

These proof-of-concept studies have led several groups to investigate the relevance of measuring intracellular CNI concentrations. Many analytical methods have been developed for this purpose and applied to various types of SOT.[12,13,21–24] However, measurement of the intracellular CNI concentration is not without preanalytical and analytical pitfalls, and standardization is critical. To date, guidelines for the TDM of intracellular CNI concentrations are not available. Moreover, no studies have been published with the aim of helping investigators conduct the necessary preanalytical and analytical components of intracellular CNI measurements.

Under the auspices of the International Association of TDM and Clinical Toxicology (IATDMCT) and its ISD committees, a group of leading investigators in this field have shared experiences and, in this article, present preanalytical and analytical recommendations for the measurement of intracellular CNI concentrations.

MATERIALS AND METHODS

Five European institutions and 1 US institution, all members of the IATDMCT and its ISD committees (Cliniques universitaires Saint-Luc, Université catholique de Louvain, Brussels, Belgium; Rennes University Hospital, Rennes, France; Oslo University Hospital, Oslo, Norway; Erasmus MC, University Medical Center Rotterdam, The Netherlands; Amedeo di Savoia Hospital, Università di Torino, Italy; and School of Pharmacy, University of Pittsburgh, Pittsburgh, United States) collaborated in this study. The recommendations were elaborated according to the Delphi method[25] and were conducted in the following manner. Concerns regarding preanalytical and analytical issues were separated into 6 themes: (1) sampling, (2) cell separation, (3) sample purification, (4) cell numeration, (5) storage, and (6) sample treatment [extraction and liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis]. Each participating center was asked to list specific issues related to the 6 themes (each expressed as a question). Nineteen questions were listed during the first round. A second round was then organized, allowing new questions to be added after considering the questions raised in the first round. Three supplementary questions were added during the second round. Thereafter, each center was asked to answer each of the questions listed for each of the 6 themes. These answers were then summarized, and recommendations were made with the approval of each participating center. The questions have been listed in **Supplemental Digital Content 1** (see **Table 1**, http://links.lww.com/TDM/A412).

RESULTS

Sampling

Is There a Risk of Active or Passive Diffusion of CNIs From PBMCs Before the Sample Reaches the Laboratory?

Preliminary results presented at the 15th Congress of IATDMCT that showed a lack of TAC back-diffusion appeared reassuring for TAC.[26] However, passive drug efflux should be further evaluated considering that TAC concentrations have been reported to be higher in PBMCs than in whole blood.[19] CNIs can thus be distributed outside PBMCs. The use of drug transporter inhibitors, particularly P-gp inhibitors, may be proposed, but is not recommended to date.[27] Active transport can also be minimized by lowering the temperature of the sample once PBMCs are separated.

Should We Ensure a Particular Temperature Threshold and a Time Limit From Sampling to the Laboratory?

Transportation from the clinical unit to the laboratory at room temperature is acceptable because there is at present no

evidence of a risk of significant passive efflux leading to a bias in determining intracellular drug concentrations. However, because of the uncertainty in time dependency of drug efflux, PBMC separation should be completed as soon as possible, and transportation time should be minimized. Thus, the time from collection to the beginning of the separation step should ideally be no longer than 4 hours [note: for blood samples collected directly into the cell preparation tube (CPT) (vacutainer), refrigeration has to be avoided]. Passive drug efflux during prolonged preanalytical transport to the laboratory (>4 hours) should be further evaluated (Fig. 1).

Which Volume of Patient Blood is Needed to Perform Cell Separation With Good Quality?

At least 3–4 mL of fresh blood should be drawn. Because no comparison between different devices has been made, a heparinized or an EDTA tube or, alternatively a dedicated, separation CPT can be used (note: the correct volume of blood should be used with each kind of the CPT, eg, 4 mL for pediatric tubes).

Cell Separation

What Kind of Device Should be Used Preferably for Cell Separation and Why?

There is no recommended device for performing cell separation, and no comparison between the available devices has been conducted so far. The panel uses Leucosep tubes (Greiner bio-One, Kremsmünster, Austria), SepMate tubes (Stem Cell Technologies, Vancouver, Canada), and CPTs (Becton Dickinson, Franklin Lakes, NJ). The listed advantages of these devices include a long experience of already being used in other applications, ready to use, high cell recovery, rapid separation with the use of brake during centrifugation, and low contamination with red blood cells.

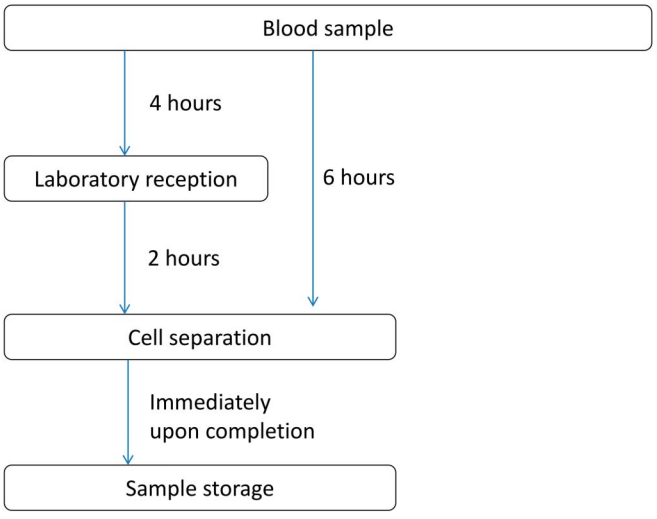

FIGURE 1. Flowchart of the recommended steps and timeline from blood collection to sample storage.

What is the Mean Cell Separation Recovery Reached With this Device?

A recovery of 50%–80% of PBMCs is usually reported when devices are used according to the manufacturer's recommendations (temperature ranging from 4°C to room temperature (15–25°C) and centrifugation at 850*g*–1800*g*). Any process with a purity of <50% PBMCs should not be accepted.

What are the Preferred Conditions to Separate Cells?

Samples should be diluted 1:1 with phosphate buffered saline (PBS). Speed, duration of centrifugation, and temperature depend on individual manufacturer's recommendations for the devices used. Further centrifugation or washing steps should be conducted at 4°C to limit the risk of diffusion. However, preliminary evidence has been presented suggesting that separation steps may be conducted at room temperature and that the risk of passive diffusion is low.[26]

What is the Maximum Recommended Duration From Sampling to Cell Isolation?

Cell separation should be completed as fast as possible. A proposed time limit between blood sampling and cell isolation is a maximum of 6 hours (note: recommended time from sampling to the beginning of separation is 4 hours, and the separation step itself should be completed within 2 hours) (Fig. 1). The time dependence of recovery during these procedures (and of subsequently reported drug concentration) should be further evaluated because no data exist at present on this factor.

Should Other Cell Populations Than PBMCs be Targeted?

Measurement of CNIs close to their site of action is ideal. However, for practical reasons, the PBMC fraction is currently the matrix of choice for intracellular drug determinations. PBMCs mainly consist of lymphocytes and monocytes with little contribution from other cell subsets. Within PBMC samples, the T-cell fraction usually represents 10%–30% of total cells. Use of PBMCs allows for easy and low-cost preparation and higher isolation yields, as well as higher sensitivity for quantitation. Most of the work published on the measurement of intracellular concentrations of CNIs has been conducted using PBMCs. The literature on measuring CNI concentrations in lymphocytes is limited and is even more limited for subfractions such as $CD4^+$ cells; however, this approach is expected to expand in the future.[20,28,29] However, analytical methods with higher sensitivity are needed to measure CNIs in lymphocytes. Moreover, active transport may differ between cell populations, and this should be taken into consideration while measuring CNIs. To date, data comparing drug concentrations in PBMCs with other subsets of white blood cells are limited. In a small study with 20 samples aimed at comparing intracellular concentrations in PBMCs, T $CD4^+$ cells, and B $CD19^+$ cells, no correlation was observed between TAC concentrations in all 3 compartments.[29] Another recent study of TAC in T cells and

PBMCs in 12 healthy volunteers also reported no correlation.[30]

Sample Purification

Is There a Need to Purify Samples Before Separation? Why and How?

Contamination by granulocytes might occur and induce biases to the measurement. Therefore, the purity of PBMC samples should at least be evaluated during the process. Alternatively, purification of the sample is possible using antibodies directed against granulocytes, but this procedure increases costs and involves additional steps.[13,31]

Is There a Need to Purify Samples After Separation? Why and How?

Erythrocyte lysis is recommended during the preparation (note: most of the experts recommend the use of a red blood cell lysis buffer).

Is There a Risk of Bias Because of Unpurified Samples?

A high risk of bias because of unpurified samples can be assumed, particularly in the presence of red blood cell contamination. Erythrocyte lysis that is usually performed using a lysis buffer (eg, buffer erythrocyte lysis, Qiagen; as proposed by Tron et al[31]) should be conducted during the preparation (and the efficiency of the lysis process should be confirmed). It should be noted that the process may be operator-dependent, and the involvement of an experienced laboratory technician is critical.

How Many Cycles of Cells Washing are Recommended?

At least 2 washing cycles with PBS are recommended to adequately purify the samples. In addition, supernatant should be analyzed to demonstrate the absence of extracellular CNIs because extracellular CNIs could result in an overestimation of the intracellular concentration.

Which Washing Buffer is Recommended?

Cold PBS (without Mg^{2+} and Ca^{2+}) or ice-cold sodium chloride 0.9% is recommended.

What are the Modalities of These Washing Cycles?

Washing cycles should preferably be performed using low-speed centrifugation (most centers reported centrifugation at 250*g*–350*g*) at 4°C.

Is There a Risk of Passive Diffusion of CNIs From PBMCs During the Purification Process Even if the Whole Procedure is Performed at 4°C?

If the washing procedure is conducted at 4°C, the results so far indicate that passive diffusion is limited. Nonetheless, a lack of remaining CNIs in the final washing supernatant evaluated using an LC-MS/MS method with sufficient sensitivity will confirm the absence of passive diffusion, and this fraction should preferably be assessed during method validation. It is recommended that the risk of passive diffusion be evaluated by using the lowest possible volume of washing buffer during the last washing cycle to evidence the lowest possible amount of drug. However, the absence of measurable drug only means that the concentration of CNIs in the wash is below the limit of quantification (LOQ) (and should be interpreted according to the washing volume used).

Is There a Need of Blocking Active Efflux?

To date, and considering the lack of comparison between the procedures, adding an active efflux inhibitor is not recommended during cell separation, although the use of a P-gp inhibitor can be considered.

Cell Counting

How Should Cell Counting be Performed?

No particular method for cell counting is recommended, although the use of an automated cell counter should be acceptable. Automated systems are more reproducible and can assess the mean cell volume (MCV) of PBMCs.[32] Upstream evaluation of sample contamination by non-PBMCs (such as granulocytes and platelets) and cell viability assessment might reduce variability in results.

How Should the Samples be Prepared After Cell Counting?

Normalization of the results is recommended. Aliquots should preferably be normalized to a fixed number of cells or within a validated range to reduce the bias. Preparations should aim for at least 500,000 cells; whereas a higher number of cells might increase the matrix effect.[33] Normalization of the results using a PBMC-derived MCV may decrease variability in the results, and its evaluation should be encouraged.[23] Normalization to the total protein cell content has also been proposed and shows a moderate correlation with normalization to the cell number.[24] Although these 2 normalization processes are not broadly used, these processes may help overcome inter-individual variability in the PBMC size; however, these procedures require further validation.

Storage

What are the Storage Specifications?

Samples, as aliquots (pellets or suspensions) containing a fixed number of cells, should be stored at temperatures ≤−70°C until further testing.

In Which Time Range Should the Isolation Process be Performed (From Cell Separation to Aliquots Storage) to Avoid Any TAC Leakage From Cells?

It is recommended that samples should be stored as soon as possible and ideally within 2 hours after cell separation (note: recommended time from sampling to the beginning of separation is 4 hours, and recommended time from sampling to the end of the separation step is 6 hours) (Fig. 1).

Sample Treatment (Extraction and LC-MS/MS Analysis)

How Should Homogenization and Cells Burst in the Sample be Ensured?

Cell lysis and the homogenization procedure should be conducted using a lysis solvent (eg, 100% methanol, 100%

water, or a 70/30% methanol/water mixture). An additional step of shaking or sonication would help the cells to lyse.

What Kind of Extraction Process is Recommended?

Several different extraction processes may be used, and no recommendation is proposed. Some of the extraction processes are described in detail in scientific publications.[13,22,34,35] Sample preparation and analysis should be conducted properly as per standard procedures, and the whole method should be validated according to international guidelines, with some added steps proposed in the present consensus.[36] The use of a liquid chromatographic method with tandem mass spectrometry detection is recommended to determine intracellular CNI concentrations.[36]

How Should the Results be Presented?

Concentrations should be normalized according to a fixed number of cells (eg, picogram per 10^6 cells) or provided as an amount per volume (eg, nanogram per milliliters or microgram per milliliters) using MCV correction.

What is the recommended lower LOQ (LLOQ) for the measurements of intracellular CNI concentrations?

To adequately measure intracellular levels of CNIs in patients (including patients with CNI minimization protocols), an LLOQ of 5 pg/10^6 PBMC or 18 ng/mL is recommended for TAC, and an LLOQ of 250 pg/10^6 PBMC or 0.9 mcg/mL is recommended for CsA.

CONCLUSIONS

The TDM of intracellular concentrations of CNIs is at an early stage of development. Measurement of the drug concentration closer to its site of action (ie, in the PBMC fraction which is enriched in lymphocytes) offers potential advantages over measuring the drug concentration in the whole blood. The proof of concept of the relevance of this approach in preventing AR has been highlighted in 1 study in liver transplant patients. However, when implementing such a strategy, there are several pitfalls that should be avoided. The present recommendations written by IATDMCT experts from 5 European and 1 US institutions are aimed at harmonizing procedures and protecting other groups from the pitfalls commonly encountered in performing intracellular drug measurements. Caution is warranted at every step of the process (from sample collection to drug assay) to reduce overall bias. Cell purification, numeration, and normalization are also crucial steps of the separation process and should be conducted in accordance with the present recommendations (because they may also lead to potential bias in intracellular CNI measurements).

At present, this approach is still relatively challenging and requires fully validated liquid chromatographic methods with tandem mass spectrometry detection of sufficient sensitivity. Indeed, the drug amount available in PBMCs is low, and in view of the trend to aim for treatment regimens based on CNI minimization, it is expected that these concentrations may be even lower in the near future. An additional challenge is the collection of a sufficient number of cells in patients treated with T-cell depleting antibodies as induction therapy. In these patients, only a limited number of PBMCs are available to conduct the procedure, making it particularly difficult, and necessitating the use of very high sensitivity LC-MS/MS equipment. Moreover, in such cases, the proportion of T cells within PBMC isolates may be lower, and this could also increase the bias. Another step aimed at reinforcing the consistency between results obtained in different centers, would be to organize a comparison of methods measuring CNI intracellular concentrations between the different centers. Using our standardized approach, future perspectives on the clinical evaluation of intracellular concentrations of CNIs as a tool to optimize drug therapy are now opened.

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