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Research Article

Spuriously low immunosuppressant results due to incomplete hemolysis – A pitfall in transplant patient therapeutic drug monitoring

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A B S T R A C T

Objective: Therapeutic drug monitoring (TDM) plays a crucial role in transplantation medicine when it comes to immunosuppressants like Tacrolimus, Cyclosporine A, Sirolimus, and Everolimus. The analysis involves using immunometric or mass spectrometric methods on whole blood samples. Hemolysis of the samples is necessary for the assessment. Typically, this is accomplished through manual protein precipitation using pre-treatment reagents, followed by vigorous vortex mixing and subsequent centrifugation. It is important to note that omitting the vortex step in these manual procedures can be seen as a potential procedural error.
Methods: To assess the potential impact of omitting the vortex step, an experiment was conducted. Clinical samples were divided into two aliquots, which were then analyzed comparatively. In one group of aliquots, the vortex step was intentionally omitted, while the other followed the correct execution of the test.
Results: The non-vortex-mixed samples showed significantly erroneous low results for all analytes.
Conclusion: Omitting or inadequately performing vortex mixing during the hemolysis procedure can be considered as a significant potential source of analytical error in TDM of immunosuppressants.

Introduction

Therapeutic drug monitoring (TDM) of immunosuppressants is a crucial aspect of transplantation medicine. The analysis of Tacrolimus, Cyclosporine A, Sirolimus (Rapamycin) and Everolimus in whole blood using tandem mass spectrometry has been widely employed in clinical laboratories for over two decades. Mass spectrometric analysis has largely replaced immunological measurement methods in this field.

When it comes to TDM of immunosuppressants, the analytes are typically measured from the sample matrix of whole blood. This is because the immunosuppressants circulate in erythrocytes or are bound to erythrocyte membranes. Serum or plasma levels of immunosuppressants are not relevant for TDM as they are significantly lower than whole blood levels. In the central laboratory, whole blood is only utilized for a limited number of analyses, with hemoglobin A1c (HbA1c) and immunosuppressants being notable exceptions.

In the chromatographic determination of HbA1c, it is important to note that this is a ratio analysis. Instead of using an absolute signal (like a UV peak area), the result is generated from a peak area ratio. In both HbA1c determination and immunosuppressant measurement, the erythrocytes are lysed during sample preparation. While incomplete lysis may not necessarily impact the results of HbA1c measurement (as it involves determining an analyte ratio of HbA1c to total hemoglobin), complete hemolysis is crucial for accurate measurement of immunosuppressants, regardless of the subsequent analytical method employed.

In most assays for immunosuppressant measurement, complete hemolysis is typically achieved through a process involving precipitation with organic solvents and salts, followed by vigorous vortexing for at least 20 s and high-speed centrifugation in reaction vessels. This results in the formation of a solid protein pellet and a clear supernatant, which is often transferred directly into a high performance liquid chromatography (HPLC) vial for subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis without further dilution [1–4]. To streamline the preparation process, an increasing number of laboratories are adopting (partially or fully automated) microtiter plate-based methods, where the protein pellet is generally left in the wells. An alternative method involves lysis of erythrocytes with distilled water prior to protein precipitation [5]. However, many published methods, widely used in laboratories, involve a single-step addition of the precipitation medium (containing an organic solvent, and in some cases zinc sulfate), in which the corresponding internal standard substances are also dissolved.

It is reasonable to assume that incomplete hemolysis could result in falsely low immunosuppressant measurements. This represents a

Abbreviations: HbA1c, hemoglobin A1c; HPLC, high performance liquid chromatography; ISD, immunosupressive drug(s); LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; QC, quality control; TDM, therapeutic drug monitoring.

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possible, relevant and potentially serious source of error in the measurement of immunosuppressants. To the best of our knowledge, this specific issue has not yet been described in the published literature.

In order to substantiate our assumption, we carried out and evaluated an analytical "provocation experiment".

Methods

For our study, we used a sample series of residual material from routine analysis to measure Tacrolimus, Cyclosporine A, Sirolimus, or Everolimus. These samples (4 randomly selected sample per analyte) had no further use in patient care and were anonymized. The use of residual material in our study follows the guidelines of the Ethics Committee of the Ludwig-Maximilians University Munich.

In our laboratory, we have been using a multianalyte method since 2000. For precipitation, we use a solution of four parts methanol and one part zinc sulfate (final concentration 61 mM), along with three internal standard substances (Ascomycin for Tacrolimus, labeled Cyclosporine A (D12), and labeled Everolimus (D4) for Everolimus and Sirolimus).

To analyze each sample, 150 μL of carefully re-suspended whole blood sample is placed in a 2 mL reaction vial. Then, 150 μL of the precipitation mix is added using a dispenser pipette. The mixture is vortexed for 20 s.

We divided our study samples equally. One aliquot of each sample was analyzed according to standard protocol with the vortex step. Another aliquot was intentionally analyzed without the vortex step, following the usual centrifugation directly after adding the precipitation medium. This intentional deviation from the correct procedure was done for both patient samples and control samples at four different concentration levels (Chromsystems, Gräfelfing, Germany; reconstituted lyophilisates), but not for calibrators.

Both the supernatants of the samples prepared with the correct procedure and the samples intentionally processed with the protocol violation were measured in the same analysis run in a series using the LC-MS/MS system.

For quantification, we used calibrator samples that were treated according to the correct procedure. As usual, the peak shape, background signal, and multiple reaction monitoring (MRM) peak area of the internal standard in all samples were evaluated during the release of analytical-technical results.

Results

The immunosuppressant results obtained from samples processed according to the correct procedure (lege artis) and those processed with the specified protocol violation are compared in Table 1. The table also includes the peak areas of the corresponding internal standards.

The comparison clearly indicates that the samples processed without vortex mixing resulted in significantly lower immunosuppressant levels compared to the correctly processed samples, with an average reduction of 70 %. This difference was statistically significant, as confirmed by a paired *t*-test. Importantly, there was no noticeable difference in the MRM peak areas of the internal standards between correctly processed and intentionally protocol-violating samples. The peak shape also appeared normal in the non-vortexed samples. Additionally, the quality control (QC) samples processed without vortex mixing did not indicate any protocol violation, as the results fell within the specified acceptance range.

Upon close visual inspection, the supernatant of the non-vortex processed samples showed a slight turbidity after centrifugation, unlike the completely clear supernatant in the vortex processed samples (Fig. 1). However, this observation can only be made in completely transparent HPLC vials made of glass, and it is effectively impossible when working with polypropylene microtiter plates.

Table 1
Results for the measurement of immunosuppressants in whole blood samples: Comparison of values when handled correctly or when the protocol is violated by omitting a vortex step.

	Result [ng/mL] – correct handling	Result [ng/mL] – without vortex mixing	Internal standard area [arbitrary units] – correct handling	Internal standard area [arbitrary units] – – without vortex mixing
Tacrolimus				
Sample #T1	4.0	1.9	22,491	20,947
Sample #T2	7.9	2.4	24,291	21,061
Sample #T3	17.7	4.8	16,672	15,190
Sample #T4	4.0	1.1	24,670	20,254
QC 1	2.9	2.2	22,398	38,741
QC 2	7.9	5.8	25,128	40,878
QC 3	16.7	14.9	25,050	35,827
QC 4	36.4	34.0	24,648	32,787
Everolimus				
Sample #E1	5.9	1.2	4805	3974
Sample #E2	9.3	1.5	3543	4362
Sample #E3	6.5	1.8	3645	4301
Sample #E4	3.8	1.2	3788	4431
QC 1	3.4	2.3	3974	7625
QC 2	5.9	4.1	4362	8125
QC 3	12.2	9.8	4301	7320
QC 4	40.9	36.0	4431	6896
Sirolimus				
Sample #S1	6.4	0.8	3663	4574
Sample #S2	3.6	0.4	3413	5720
Sample #S3	4.8	0.8	4405	5416
Sample #S4	5.7	1.0	4307	4596
QC 1	3.8	3.1	3974	7625
QC 2	14.3	11.1	4362	8125
QC 3	28.8	28.1	4301	7320
QC 4	55.9	57.7	4431	6896
Cyclosporin A				
Sample #C1	194.8	118.1	121,604	80,059
Sample #C2	335	178.7	118,262	84,664
Sample #C3	221	84.4	111,345	86,601
Sample #C4	98.7	42.4	109,162	83,511
QC 1	57.5	36.6	106,402	126,272
QC 2	285.4	190.6	116,186	133,729
QC 3	568.1	424.2	117,246	119,137
QC 4	1370	1114	112,286	104,377

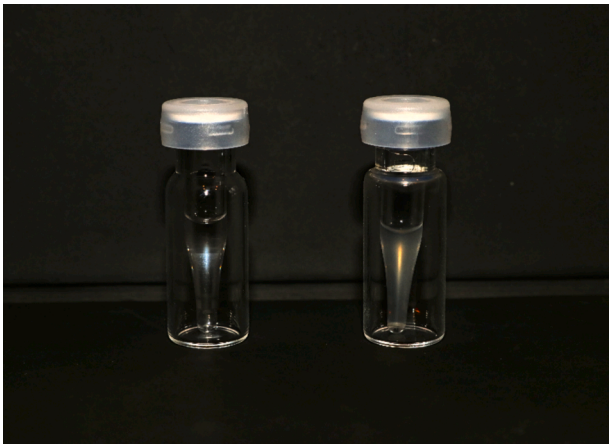

Fig. 1. Protein-precipitated whole blood samples after centrifugation with previous vigorous vortex mixing (left) and without vortex mixing (right).

Discussion

Our findings strongly support the conclusion that insufficient hemolysis, due to the omission of vortex mixing, leads to significantly underestimated results when measuring immunosuppressants in our model. Interestingly, our evaluation of the MRM chromatograms and internal standard recovery did not provide any indication of the deliberately induced processing error.

It is worth noting that the QC samples did not reveal the protocol violation either. This can be explained by the fact that these samples are always hemolysates, which are essentially not affected by handling conditions that do not lead to hemolysis of erythrocytes.

In summary, our simple provocation experiment confirms the critical importance of complete hemolysis in analytical procedures for determining immunosuppressants. This factor should be considered independent of the signal generation and quantification method, and should be expected in both immunometric and mass spectrometric methods.

Depending on the specific analysis process used in a laboratory, there can be various errors that may result in falsely low results due to inadequate hemolysis. This is particularly relevant when the vortex step is accidentally overlooked for certain samples during processing, leading to selective carelessness – i.e., where the sample is directly transferred to the centrifuge after adding the precipitation medium. It is also conceivable that the vortex mixing step is omitted for an entire batch of samples.

To detect falsely low results caused by insufficient protein precipitation, trend analysis of measurement results can be performed by comparing patient results with previous individual results. If all samples in a run consistently show a suspicious difference compared to the corresponding previous results, this could indicate inadequate protein precipitation.

Further, it can be concluded from our results that the completeness of hemolysis must be carefully verified when developing methods for measuring immunosupressive drugs (ISD), or other target analytes, in whole blood. To the best of our knowledge, this has not yet been sufficiently emphasized in the literature. This applies to both the vortex and shaking protocol (intensity, duration, volume ratios) on the one hand, and the precipitation media used on the other. Since internal and external quality assurance samples are available as lyophilized hemolysates only, their sole use is not sufficient for method validation. It is assumed that the error of "incomplete hemolysis" in the ISD measurement can occur gradually. Therefore, in all corresponding protocols – whether manual or automated – standardized hemolysis steps must be carefully followed. In automated sample preparation protocols performed on pipetting platforms, the failure of the shaking device (even partially) may not be obvious and could therefore go undetected, resulting in falsely low immunosuppression results. Visual detection of slight turbidity in centrifuged samples to indicate incomplete hemolysis before centrifugation is not reliable, especially when using microtiter plates. Having a biomarker to confirm the completeness of hemolysis as a process control, which is evaluated simultaneously with the analysis run, would be desirable. However, the authors are not aware of any corresponding approach. Further research for this purpose is warranted.

LC-MS/MS generally enables analyses at a very high level of reliability in the clinical laboratory, but a range of potential sources of error must be considered [6].

In summary, our short article aims to highlight incomplete hemolysis as a significant potential source of error in TDM of immunosuppressants. This is directly relevant to patient safety, as inaccurately low immunosuppressant measurements can lead to dose escalation and cause significant harm to the patient.

Ethics statement

Only anonymised residual samples that were no longer of use to the patients were used for this study. No additional sampling was carried out for the study. This procedure complies with the requirements of the Ethics Committee of the Ludwig-Maximilians-Universität München, Germany.

CRediT authorship contribution statement

Michael Vogeser: Writing – original draft, Supervision, Project administration, Data curation, Conceptualization. **Katharina Habler:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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