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Validation and clinical application of an LC-MS/MS method for the quantification of everolimus using volumetric absorptive microsampling

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A B S T R A C T

Everolimus is a mammalian target of rapamycin inhibitor approved for the treatment of various tumor types. Less invasive measurement of everolimus concentrations could facilitate pharmacokinetic studies and personalized dosing based on whole blood concentrations, known as therapeutic drug monitoring.

Volumetric Absorptive Microsampling (VAMS) has been introduced as a patient friendly, less invasive sampling technique to obtain an accurate volume of whole blood regardless of hematocrit value. We describe the bioanalytical validation and clinical application of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify everolimus using VAMS.

For the quantification, $^{13}C_2D_4$-Everolimus was used as internal standard (IS). Everolimus and the IS were extracted with methanol from the VAMS device, which was evaporated after ultrasonification and shaking. The residue was reconstituted in 20 mM ammonium formate buffer and methanol (50%, v/v) of which 5 µL was injected into the LC-MS/MS system. Quantification was performed for the ammonium adduct of everolimus in positive electrospray ion mode.

The VAMS method met all pre-defined validation criteria. Accuracy and precision were within 11.1% and ≤14.6%, respectively. Samples were shown to be stable on the VAMS device for at least 362 days at ambient temperatures. Considerable biases from −20 to 31% were observed over a 30–50% hematocrit range.

Although the method fulfilled all validation criteria, the perceived advantage of VAMS over dried blood spot sampling could not be demonstrated. Despite the effect of hematocrit, using an empirically derived formula the whole blood everolimus concentration could be back calculated with reasonable accuracy in the clinical application study.

1. Introduction

Everolimus is a mammalian target of rapamycin (mTOR)-inhibitor approved for the treatment of renal cell carcinoma [1], neuroendocrine tumors [2] and hormone receptor positive, human epidermal growth factor receptor 2 (HER2) negative, breast cancer [3]. In all these tumor types, everolimus is currently administered using a 10 mg once daily oral dosing regimen. Yet in transplantation medicine (where everolimus is used as an immunosuppressant) personalized dosing based on measured blood concentrations, known as therapeutic drug monitoring is applied routinely [4]. Increasingly, personalized dosing of everolimus is also being advocated for in oncology [5–7]. A possible hurdle to the implementation of therapeutic drug monitoring could be the need for additional invasive blood sampling to enable drug concentration measurements. Dried blood spots (DBS) have been proposed as a patient friendly, less invasive alternative to standard blood sampling [8,9] and have been applied to the quantification of everolimus [10–12]. However, analyses on DBS need to be validated using additional test to specifically investigate the influence of hematocrit, spot volume and other factors such as sample homogeneity on the analytical results [13].

Volumetric Absorptive Microsampling (VAMS) has been introduced as an alternative dried blood sampling technique specifically designed to overcome these perceived disadvantages [14]. Specifically, it has been shown that using the VAMS method exactly 10 µL samples could be collected and the influence of hematocrit was reduced, if not completely eliminated for selected analytes [14,15]. Everolimus is an ideal candidate drug for VAMS sampling as everolimus is normally measured in whole blood and methods using DBS have demonstrated a clear influence of hematocrit [12].

Given these theoretical advantages, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantification of everolimus using VAMS was developed and validated. After the bioanalytical validation, the analytical performance of the VAMS system was investigated over a range of hematocrit values. Finally, the

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VAMS method was applied to a collection of clinical samples for pharmacokinetic measurements in cancer patients treated with everolimus.

2. Materials & methods

2.1. Chemicals

Everolimus and stable isotopically labeled internal standard (IS) $^{13}C_2D_4$-Everolimus were supplied by Alsachim (Illkirch Graffenstaden, France). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany), ammonia (Empure® 25%) and methanol (UPLC grade) from BioSolve Ltd. (Valkenswaard, The Netherlands). Control human EDTA whole blood was obtained from healthy volunteers and used for preparation of quality control samples (QC), calibration standards and matrix blanks. Mitra®, VAMS devices were obtained from Neoteryx, LLC (Torrance, CA, USA).

2.2. Stock solutions, calibration standards and quality control samples

Stock solutions of everolimus were prepared in DMSO at a concentration of 1 mg/mL. Working solutions were prepared by diluting stock solutions with methanol. The IS stock solution was prepared in methanol at a concentration of 1 mg/mL. The IS working solution was prepared by further dilution with methanol to a concentration of 10 ng/mL. All stock and working solutions were stored at −20 °C.

Calibration standards and QC samples were prepared by addition of a 10 µL aliquot of working solution to 190 µL of control whole blood, of which 10 µL was subsequently absorbed using the VAMS device. Nominal concentrations of 2.50, 7.50, 25.0 and 80.0 ng/mL were used for the QC samples (lower limit of quantification (LLOQ), Low, Mid and High concentrations, respectively) and 2.50, 5.00, 10.0, 25.0, 50.0, 75.0, 90.0 and 100 ng/mL for the 8 calibrations standards.

2.3. Liquid chromatography tandem mass spectrometry

All LC-MS/MS experiments were performed using the I-class Acquity UPLC system, consisting of a autosampler, pump and column oven by Waters (Milford, MA, USA) and a QTRAP® 5500 MS system equipped with a turboionspray, and Analyst™ software was used for data analysis by Sciex (Framingham, USA).

Chromatographic separation is performed on a Acquity BEH C18 analytical column, 100 × 2.1 mm ID, 1.7 µm particle size (Waters) using an 0.2 µm in-line filter. The column oven was set at 40 °C and the autosampler tray at 8 °C. Elution was achieved using a mixture of 20 mM ammonium formate in water (eluent A) and methanol (eluent B) at flow of 0.4 mL/min. The gradient would start at 50% methanol and would rise linearly to 95% methanol from 0.20 to 0.45 min. After 1.5 min the gradient would return to 50% methanol, until the end of the run at 2.0 min.

Final optimized MS settings for these were 5000 V for the ion spray voltage, 350 °C for the ionization temperature, 25 and 7 arbitrary units for the curtain gas and collision, gas respectively. Declustering potential was set at 56 V, collision energy at 31 V, collision cell exit potential at 40 V and entrance potential at 10 V. Quantification was performed on the ammonium adduct of everolimus [11,12] in positive ion mode using the m/z 975.6 → m/z 908.8 transition for everolimus and m/z 981.6 → m/z 914.5 for $^{13}C_2D_4$-everolimus. The chemical structures of everolimus and $^{13}C_2D_4$-Everolimus are provided in Fig. 1.

2.4. Sample preparation

The tip of the sampling device was transferred to an Eppendorf tube of 2.0 mL. A volume of 10 µL of IS working solution and 500 µL of methanol were added and the sample was vortex mixed. The samples were ultrasonicated for 5 min and shaken at 500 rpm for 5 min. Methanol was then evaporated under a gentle stream of nitrogen. The residue was reconstituted in 50 µL of reconstitution solvent (20 mM ammonium formate: methanol, 1:1, v/v), vortexed and centrifuged for 3 min at 15,000 rpm. Finally, the solution was transferred to an autosampler vial and 5 µL was injected into the LC-MS/MS system.

2.5. Bioanalytical validation

The bioanalytical method validation was conducted in accordance with guidelines for bioanalytical method validation by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) [16,17]. The following validation parameters were assessed: calibration model, accuracy and precision, LLOQ, dilution integrity, selectivity, instrument carry-over, matrix factor, recovery and stability in final extract and on the dried blood sample using the VAMS device.

2.6. Calibration

Weighted linear regression (1/concentration^2) was applied to fit the calibration curves (area ratio vs square of the concentration). At least 75% of the non-zero standards (including at least one LLOQ and upper limit of quantification (ULOQ)) in each run had to be within ± 15% of the nominal value (± 20% for the LLOQ). For the LLOQ and ULOQ levels at least 50% had to meet these criteria. The regression coefficient was calculated for each analytical run.

2.7. Accuracy and precision

Accuracy and precision were determined in three separate validation runs by injecting five replicates of QC samples at the LLOQ, Low, Mid and High concentrations. Intra-run and overall accuracy were expressed as the relative bias. The intra-run and overall precision were calculated as the coefficient of variation (CV). At each concentration level, the bias had to be within ± 15% and the precision ≤15%. For the LLOQ concentrations bias had to be within ± 20% and the precision ≤20%.

2.8. LLOQ

The LLOQ of the method was evaluated in each analytical run. It was quantified as the ratio of the peak height of the 2.50 ng/mL calibration standard (the signal) to the peak height of a double blank sample (the noise). A predefined limit of ≥5 was set for this ratio.

2.9. Dilution integrity

The dilution integrity was studied by analyzing five replicate samples at concentration of 150 ng/mL. These samples were diluted 2 times with final extract from a blank sample (to which IS has been added before processing). Predefined limits for bias and precision were set at ± 15% and ≤15% respectively.

2.10. Selectivity

The selectivity of the assay was determined for cross analyte/IS interference and possible endogenous interferences.

The cross analyte/IS interference was assessed by analyzing an everolimus ULOQ sample without adding the IS and by spiking IS separately to a double blank sample at the concentration used in the assay. The analyte/IS interference was considered acceptable if it was ≤20% of the response of the LLOQ of the analyte and ≤5% of the response of the IS.

The possibility of endogenous interferences was assessed by analyzing double blank samples from six different individuals and comparing the peak area in the blank with the peak area of the LLOQ in the same analytical run.

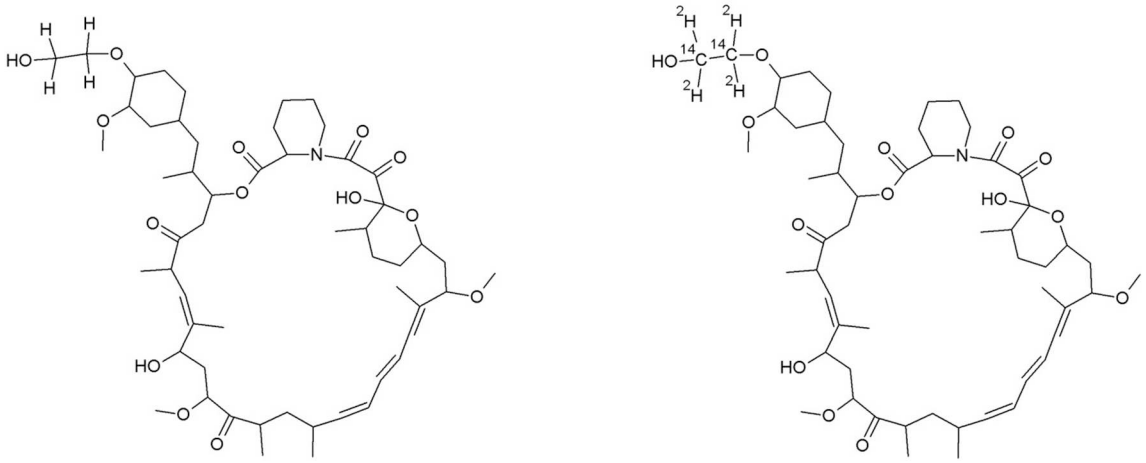

Fig. 1. The chemical structures of everolimus and $^{13}C_2D_4$-Everolimus.

The endogenous and IS interferences were considered acceptable if it was ≤20% of the response of the LLOQ of the analyte.

2.11. Instrument carry-over

The instrumentation carry-over was tested by injecting two double blank samples after an ULOQ sample in each validation run. The carry-over was calculated as the ratio of the peak area in the blanks and the peak area of the LLOQ. The carry-over was considered acceptable if the response at the retention time of the analyte (for both everolimus and the IS) was ≤20% of the response of the LLOQ in the first blank.

2.12. Matrix factor

The matrix factor (MF) was determined in six different batches of whole blood spiked at both the QC Low and QC High concentration. The MF was calculated by dividing the everolimus peak area in presence of matrix by the peak area at the same concentration in a neat solution. This was calculated for six different batches of control human whole blood and was considered acceptable if the coefficient of variation of the MF across the six batches was ≤15%.

2.13. Sample pretreatment recovery

Recovery was determined by dividing the peak area of everolimus in processed validation samples at QC low and high concentrations (n = 5) by the peak area of everolimus in presence of matrix (a double blank sample to which everolimus was spiked after processing).

2.14. Stability

The stability of VAMS samples was assessed at ambient temperatures. The stability of everolimus in processed samples (final extract) was determined after being stored at nominally 2–8 °C.

All these stability analyses were carried out in triplicate at the QC Low and High concentrations. Samples were considered to be stable if the measured concentration was within ± 15% of the nominal concentration.

2.15. Influence of hematocrit

To determine the relative influence of hematocrit on quantification, VAMS samples were analyzed in duplicate on the QC Low, Mid and High concentrations in whole blood at nominal hematocrit concentrations of 30, 40 and 50% (± 1%).

Relative deviations were calculated, normalized to the respective (Low, Mid or High) QC at a hematocrit of 40%. The hematocrit value of the calibration standards used to quantify the VAMS samples was 44%. Hematocrit measurements were performed using the Mission Plus (Acon Laboratories Inc., San Diego, USA).

2.16. Clinical application

Paired VAMS and whole blood clinical samples were obtained in a pharmacokinetic study in cancer patients. This trial was registered in the EudraCT database (2014-004833-25) and the Netherlands Trial Registry (NTR4908) [18]. This trial was conducted in accordance with the World Medical Organization declaration of Helsinki, compliant with Good Clinical Practice and approved by the Medical Ethics Committee of each of the participating medical centers. All patients provided written informed consent before enrollment.

EDTA whole blood samples were drawn by venipuncture by a trained nurse or physician. VAMS samples were taken by the patient under supervision of a researcher, in accordance with the manufacturers' instruction. VAMS samples were prepared and analyzed by the methods described in this manuscript and whole blood concentrations were quantified using a previously developed and validated LC-MS/MS method [18]. Weighted Deming regression was applied to compare the VAMS (mean of both samples) with the whole blood everolimus concentrations. Based on the empirical correlation found, the back calculated whole blood concentrations would be determined and compared to the actual whole blood concentration, as described previously [19,20].

3. Results

3.1. Validation

The method was successfully validated in accordance with the FDA and EMA guidelines and met all pre-specified acceptance criteria. An overview of the validation parameters is provided in Table 1.

3.2. Influence of hematocrit

The influence of hematocrit on the quantification of everolimus using VAMS was investigated. The results are displayed in Fig. 2. The VAMS assay showed a marked influence of hematocrit: at the lower hematocrit value (31%) relative biases were 24%, 31% and 13% for the Low, Mid and High concentration, respectively (shown as normalized response ratios of 1.24, 1.31 and 1.13 in Fig. 2). At high hematocrit values (49%) relative biases of −16%, −20% and −20%, respectively, (depicted as ratios of 0.84, 0.80 and 0.80) were observed.

Table 1
Overview of the bioanalytical validation outcome data. All tested parameters met their predefined criteria[a].

Validation parameter	Outcome
Calibration model	$1/\chi^2$ weighted linear regression, all regression coefficients > 0.99
Calibration range	2.50–100 ng/mL
Intra-run accuracy (%)	LLOQ: −16.8% Other: 11.1%
Overall accuracy (%)	LLOQ: −0.2% Other: 11.1%
Intra-run precision (CV)	LLOQ: 8.6% Other: 9.7%
Overall precision (CV)	LLOQ: 14.6% Other: 9.0%
Lower limit of quantitation (signal/noise ratio)	≥5.6
Dilution integrity (bias, CV)	−7.6%, 15.0%
Cross analyte/IS interference	Not detected
Endogenous interferences	Not detected
Instrument carry-over	Not detected
Matrix factor (CV)	QC Low: 0.640, 3.7% QC High: 0.636, 4.3%
Recovery (mean, CV)[b]	QC Low: 23.1%, 7.3% QC High: 20.2%, 9.8%
Stability in final extract at 2–8 °C after 48 h (bias, CV)	QC Low: 9.8%, 3.3% QC High: −1.9%, 6.2%
Stability of dried VAMS samples at ambient temperatures after 362 days (bias, CV)	Low: 12.1%, 0.9% High: −1.6%, 6.2%

CV: coefficient of variation; LLOQ: Lower limit of quantification.
[a] Predefined acceptance criteria are reported in the text.
[b] Recovery experiments for the purpose of the validation were performed at an hematocrit of 44%.

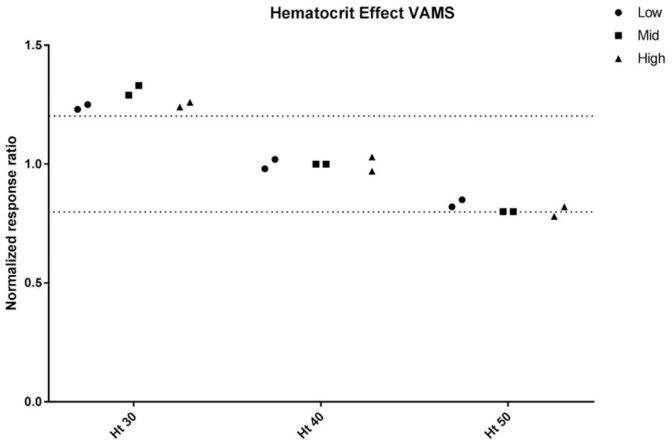

Fig. 2. Relative analytical response ratios for everolimus quantified at QC Low, Mid and High concentrations for three different hematocrit values. Analyte/internal standard response ratios were normalized to the QC at the 40% hematocrit. Dotted lines indicate ± 20% deviation.

3.3. Clinical application

Overall, 25 clinical samples were available from 10 patients. Of these, all but one were taken in duplicate. The VAMS everolimus concentrations are plotted versus the corresponding whole blood concentrations in Fig. 3. Weighted Deming regression was used to compare the VAMS and the whole blood LC-MS/MS methods. The relation was described quantitatively by the formula of y = 0.691x + 0.158, where x is the VAMS and y is the whole blood concentration, respectively. According to this formula the whole blood concentration could be back calculated based on the VAMS results. These calculated whole blood concentrations are plotted versus the actual measured whole blood concentrations in Fig. 4. The differences between the two methods in further analyzed in Fig. 5 which shows a Bland-Altman plot of the difference (plus its 95% confidence interval) between whole blood and VAMS concentrations versus the mean of the methods.

The average deviation (taken as absolute values) between the VAMS

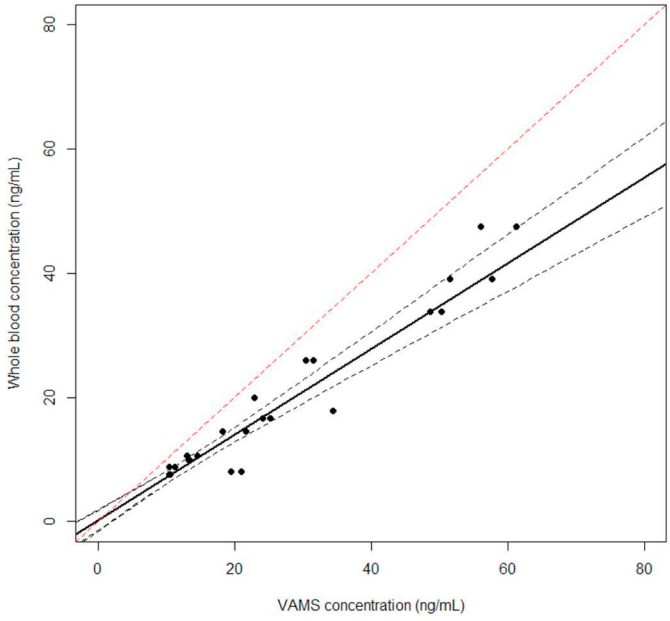

Fig. 3. Everolimus concentration in 25 clinical samples determined by volumetric absorptive microsampling (VAMS) and whole blood analysis, with weighted linear Deming regression (black line) and 95% confidence interval (dotted black line). The red dotted line indicates unity. The relation between VAMS and whole blood was described by y = 0.691x + 0.158. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

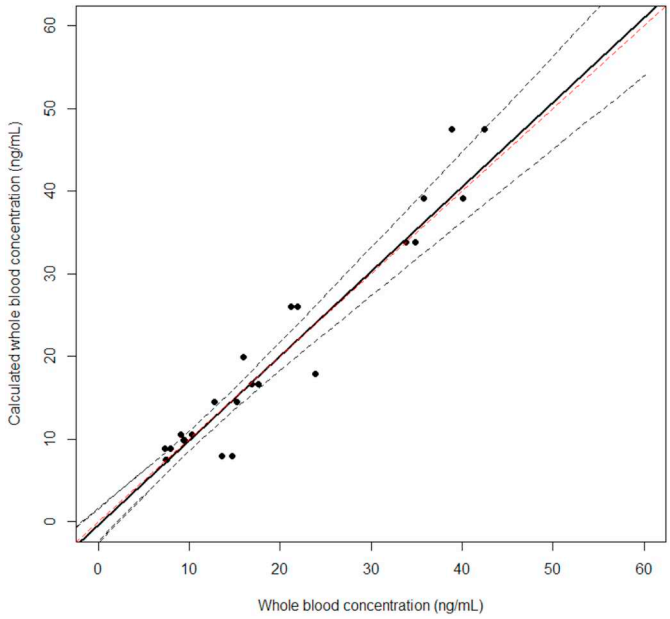

Fig. 4. Everolimus concentration in 25 clinical samples determined in whole blood compared to the back calculated whole blood concentration based on the VAMS analysis, with weighted linear Deming regression (black line) and 95% confidence interval (dotted black line). The red dotted line indicates unity. The relation between The calculated whole blood concentration was determined using y = 0.691x + 0.158 (see Fig. 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

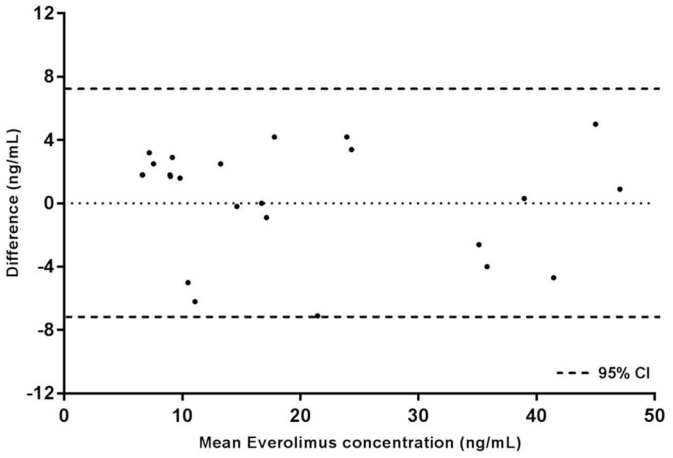

Fig. 5. Bland-Altman plot of the difference between whole blood and VAMS everolimus concentrations versus the mean of the methods (both plotted as ng/mL). The dotted lines represent the 95% confidence interval of the difference.

samples taken in duplicate was 6.4%. The bias between the calculated and actual whole blood concentrations of everolimus was 0.6% and the mean absolute difference was 14.1%. Mean (CV%) hematocrit of the patients enrolled in this trial was 36% (11%).

4. Discussion

We describe the bioanalytical validation and clinical application of an LC-MS/MS method to quantify everolimus using VAMS. The method met all pre-specified acceptance criteria although the matrix factor and recovery of the method were relative low at only 0.64 and 20.2–23.1%, respectively. The perceived major advantage of VAMS over DBS sampling is the volumetric collection of a fixed volume, which should be independent of hematocrit. However, we clearly showed a large impact of hematocrit on analytical performance.

Previous studies have found that VAMS eliminated an effect of hematocrit on the analysis of caffeine and paraxanthine [15], In the current study, biases of −20 to 31% were found over the tested hematocrit range. Comparable deviations were also found in previously published methods e.g. the quantification of miltefosine [21]. Moreover, the inversely correlated direction of the observed effect was the same, i.e. positive bias for low and negative bias for high hematocrit values. It is hypothesized that this phenomenon is due to the presence of a larger amount of erythrocytes which entrap the analyte in the pores of the VAMS device tip, hindering analyte extraction [15].

DBS analysis of everolimus has also been shown to be strongly influenced by blood hematocrit in a concentration dependent manner [12]. Interestingly, for the VAMS analysis of everolimus the hematocrit driven relative bias seemed independent of the everolimus concentration (Fig. 2), whilst a considerable effect of concentration was found for DBS quantification of everolimus at varying hematocrit values [12]. Yet, based on our data it must be concluded that no superiority of VAMS over DBS in reducing the effect of hematocrit on everolimus quantification could be demonstrated.

Everolimus concentrations in clinical VAMS samples were consistently higher than the whole blood concentrations (Fig. 3). Interestingly, the slope of the linear regression of VAMS concentrations and whole blood concentrations was only 0.691 where a slope of unity would have been expected, as both samples were from the same matrix. These results are consistent with previous reports regarding caffeine, paraxanthine and paracetamol, where VAMS systematically overestimated the whole blood concentration [15,22] The underlying explanation of this effect is not clearly understood, but in our study it could be due to the higher hematocrit level (44%) of the calibration standard compared to that of the enrolled patients (36%). Even though considerable effects of hematocrit were found for VAMS, using an empirically determined back calculation the method did result in a reasonable estimation of the whole blood concentration (Figs. 4 and 5). The current study is the first clinical validation study to use this backward calculation method based on the empirical relation for estimating whole blood concentration of everolimus using VAMS and whole blood samples. However, this calculation has been reported to be successful in DBS analysis for other small molecules both in oncology and infectious diseases such as pazopanib, vemurafenib and miltefosine [19,20,23]. A drawback of the clinical validation study is its modest size and the fact that only oncology patients, treated at a relatively higher dose, were included and therefore limited values are available in the lower concentration range expected in transplantation medicine.

A possible drawback of the VAMS could be the between-operator variability [14], but the small variation between the samples drawn in duplicate of 6.4% seem to diminish this concern. Possible advantages of VAMS over DBS sampling would be the accurate whole blood volume sampling regardless of hematocrit value and reducing homogeneity issues [14]. Based on these theoretical advantages, we developed the currently described method. However, specifically for the case of everolimus, we showed that this theoretical advantage of VAMS could not be demonstrated in practice.

5. Conclusion

We describe the bioanalytical validation of an LC-MS/MS method to quantify everolimus using VAMS. The method met all pre-defined bioanalytical validation criteria and samples were shown to be stable for nearly a year (362 days) at ambient temperatures. The analytical performance of the VAMS method was studied over a 30–50% hematocrit range, where large relative biases were found. Therefore, no superiority of the VAMS over DBS sampling was demonstrated. Despite the effect of hematocrit, using an empirically derived formula the whole blood everolimus concentration could be back calculated with reasonable accuracy in the clinical application study.

Compliance with ethical standards

This research was conducted in accordance with the World Medical Organization declaration of Helsinki, compliant with Good Clinical Practice and approved by the Medical Ethics Committee of the each participating medical center (The Netherlands Cancer Institute and Erasmus MC Cancer Institute). All patients provided written informed consent before enrollment. This trial was registered in the EudraCT database (2014-004833-25) and the Netherlands Trial Registry (NTR4908).

Funding statement

No funding was received for this research.

Conflicts of interest

N. Steeghs received funding from Novartis as principal investigator for this investigator initiated study. R.B. Verheijen is currently an employee of AstraZeneca. B. Thijssen, F. Atrafi, J.H.M. Schellens, H. Rosing, N. de Vries, J.H. Beijnen, R.H.J. Mathijssen and A.D.R. Huitema all declare they have no conflicts to disclose.

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