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Development and Validation of an Efficient and Highly Sensitive Enzyme-Linked Immunosorbent Assay for Alemtuzumab Quantification in Human Serum and Plasma

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Background: Alemtuzumab is a humanized monoclonal antibody that targets the CD52 glycoprotein expressed on most lymphocytes, subsequently inducing complement-mediated and antibody-mediated cytotoxicity. Owing to its ability to induce profound immune depletion, alemtuzumab is frequently used in patients before allogeneic hematopoietic stem cell transplantation to prevent graft rejection and acute graft-versus-host disease. In this clinical context, a stable immunoassay with high sensitivity and specificity to determine alemtuzumab levels is essential for performing pharmacokinetic and pharmacodynamic analyses; however, the available methods have several limitations. Here, we report the successful development and validation of an efficient and highly sensitive enzyme-linked immunosorbent assay technique based on commercially available reagents to quantify alemtuzumab in human serum or plasma.

Methods: This enzyme-linked immunosorbent assay technique was developed and validated in accordance with the European Medicines Agency guidelines on bioanalytical method validation.

Results: The assay sensitivity (lower limit of quantification) is 0.5 $ng \cdot mL^{-1}$, and the dynamic range is 0.78–25 $ng \cdot mL^{-1}$. To accommodate quantification of peak concentration and concentrations below the lympholytic level (<0.1 $mcg \cdot mL^{-1}$), patients' serum samples were prediluted 20–400 times according to the expected alemtuzumab concentration. The overall within-run accuracy was between 96% and 105%, whereas overall within-run precision (coefficient of variation) was between 3% and 9%. The between-run assessment provided an overall accuracy between 86% and 95% and an overall coefficient of variation between 5% and 14%.

Conclusions: The developed assay provides accurate insight into alemtuzumab exposure and its effects on the clinical response to treatment, which is key to optimizing treatment strategies.

Key Words: alemtuzumab, anti-CD52 humanized monoclonal antibody, immunoassay, pharmacokinetics

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INTRODUCTION

Alemtuzumab (Campath-1H; Sanofi Genzyme, Cambridge, MA) is a humanized monoclonal IgG1 antibody with a rat-derived antigen-specific Fab region and human Fc region.[1,2] This antibody was created to target the human

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The authors confirm that Prof. Arjan C. Lankester is the principal investigator in the Netherlands for the clinical pharmacokinetic/pharmacodynamic study (ARTIC study, NL8185/BASEC-2018-00794), which provided the patient samples for testing the applicability of this enzyme-linked immunosorbent assay in the clinical setting, and that he has direct clinical responsibility for the patients. All patients and their parents provided informed consent for participation in the study, biobanking, and medical research.

Alemtuzumab was used in this research project to evaluate its pharmacokinetic characteristics in children undergoing allogeneic hematopoietic stem cell transplantation based on the current knowledge (standard of care approach). Alemtuzumab has been used for many years for this specific indication in children, and its safety and efficacy have been detailed in peer-reviewed publications. Our article describes the measurement of alemtuzumab using an internally developed highly sensitive enzyme-linked immunosorbent assay, and clinical data are limited to the assessment of clinical feasibility.

Data supporting the findings of this study are available in the supplementary material of this article.

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CD52 antigen, a membrane protein highly expressed on the surface of T and B lymphocytes and at a lower level on monocytes, macrophages, natural killer cells, and neutrophils, but not on hematopoietic stem cells.[3,4] Alemtuzumab is used to deplete CD52-expressing cells, thereby inducing profound immunosuppression.[2] In clinical practice, indications for alemtuzumab treatment are autoimmune diseases, such as multiple sclerosis (MS);[5] severe inflammatory diseases, such as hemophagocytic lymphohistiocytosis;[6] and hematological malignancies, such as chronic lymphocytic leukemia[7] and lymphoma.[8] In addition, alemtuzumab is frequently administered to prevent graft-versus-host disease and graft rejection in patients undergoing solid organ or allogeneic hematopoietic stem cell transplantation (HSCT).[2] Alemtuzumab has a relatively long half-life in humans, which may be advantageous in some applications but can have negative effects in patients undergoing HSCT because it may delay lymphocyte reconstitution and antiviral immunity. In most patients, a standard dosing schedule is used, but owing to significant variation in clearance, this may result in variable exposure and a divergent response to treatment.[9,10] For pharmacokinetic (PK) analysis, a reliable assay to quantify alemtuzumab with optimal sensitivity is of pivotal importance. Because alemtuzumab concentrations as low as 0.1 mcg·mL^{-1} is sufficient to induce antibody-dependent cell-mediated cytotoxicity with human effector cells in vitro, thereby having a drug lympholytic level of 0.1 mcg·mL^{-1}, a sensitive assay able to detect concentrations below 0.1 mcg·mL^{-1} is required for reliable PK analysis.[1,11,12] However, published flow cytometry, enzyme-linked immunosorbent assay (ELISA), and electrochemiluminescence bridging immunoassay methods to measure alemtuzumab present several limitations pertaining to sensitivity and availability of reagents.

The primary objective of this study was to develop and validate an efficient and highly sensitive ELISA based on commercially available reagents for measuring alemtuzumab concentrations in human serum or plasma samples.

MATERIALS AND METHODS

Reagents

Alemtuzumab (30 mg·mL^{-1}; Sanofi Genzyme, Cambridge, MA) was stored at 2–8°C, away from light. Human polyclonal anti-idiotypic antialemtuzumab antibodies (1 mg·mL^{-1}), which were originally isolated from the plasma of a patient who developed antialemtuzumab antibodies, were purchased (NC; Geoff Hale Developments, Oxford, England). Human polyclonal anti-idiotypic antialemtuzumab antibodies labeled with long-chain biotin (NHS-LC-Biotin; Pierce, EZ-link) were provided by the same company at a concentration of 1 mg·mL^{-1}. All reagents were aliquoted and stored at −80°C.

Matrix Selection and Calibration Standards

ELISA technique was developed to determine alemtuzumab concentrations in the serum and plasma samples of children undergoing HSCT. Alemtuzumab was diluted in phosphate-buffered saline (PBS; Fresenius Kabi, Bad Homburg Vor der Höhe, Germany) pH 7.2 with 1% human serum albumin (HSA; 40 g L^{-1}; Sanquin, Amsterdam, the Netherlands) to provide 7 calibration standards (0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25 ng·mL^{-1}) and 2 quality controls (QCs; 1200 and 300 ng·mL^{-1}, subsequently diluted in assay buffer to 12 and 3 ng·mL^{-1}, respectively). Alemtuzumab dilutions were aliquoted and stored at −80°C. On each ELISA plate, a calibration standard and 2 QCs prepared from a different alemtuzumab batch were included in duplicate.

Alemtuzumab Sandwich ELISA/NC ELISA

Flat-bottom 96-well high-binding microtiter ELISA plates (Thermo Fisher, Waltham, MA) were coated with human anti-idiotypic antialemtuzumab antibodies (50 μL) diluted in PBS to 0.5 mcg·mL^{-1} (1:2000) over at least 1 night and maximal 7 days at 4°C. Subsequently, ELISA plates were washed 4 times with the wash buffer (PBS containing 0.05% Tween 20; Merck, Darmstadt, Germany) and blocked with 150 μL of assay buffer (PBS containing 0.05% Tween 20 and 2% bovine casein; Sigma-Aldrich - Merck, Darmstadt, Germany) for 1 hour at room temperature. ELISA plates were then washed 4 times with the wash buffer and refilled with 50 μL of calibration standards in duplicate in the assay buffer. Patient serum samples containing alemtuzumab were prediluted 20–400 times in the assay buffer, taking the expected alemtuzumab concentration into account and targeting concentrations within the concentration range defined by both QCs (3–12 ng·mL^{-1}). Samples with missing information on the expected alemtuzumab concentration were prediluted 50 times in the assay buffer. Predilutions (50 μL) were added to the wells prefilled with the assay buffer and serially diluted 2-fold in the plate. ELISA plates were incubated for 1 hour at room temperature. After washing the plates 4 times with the wash buffer, 50 μL of biotin-labeled antialemtuzumab antibodies diluted in assay buffer to 0.2 mcg·mL^{-1} (1:5000) was added to each well. ELISA plates were incubated for 1 hour at room temperature and washed 4 times. Subsequently, streptavidin poly-HRP (Sanquin) diluted to 0.1 mcg·mL^{-1} (1:10,000) in assay buffer was added to each well, and the plates were incubated for 30 minutes at room temperature. ELISA plates were washed 4 more times and refilled with 50 μL of undiluted 3,3′,5,5′-tetramethylbenzidine (0.4 g L^{-1}) colorimetric substrate (Thermo Fisher). After 10–15 minutes, the staining reaction was stopped by adding 50 μL of H_2SO_4 (1 M; Merck) to each well. Optical density (OD) was measured at 450 nm using an ELISA plate reader (VersaMax; Molecular Devices, San Jose, CA). The ODs obtained from the calibration standards were fitted to a 4-parameter nonlinear regression model (4PL, sigmoid function) to generate an S-shaped standard curve using SoftMax Pro 7 software (Molecular Devices). The R^2 value represents the goodness-of-fit of the curve. Only R^2 values above 0.98 were accepted. Based on the back-calculated concentrations in ng·mL^{-1} and mcg·mL^{-1}, only ELISA plates with QCs levels within ±20% of the theoretical concentration and samples yielding alemtuzumab concentrations within the quantifiable range of the standard curve were used for data analysis. Potential outliers were defined using the Dixon Q test and were excluded from the data analysis. All remaining values were averaged and reported in ng·mL^{-1} (validation results) and mcg·mL^{-1} (patient results) with a coefficient of variation (CV, %).

Validation Requirements

ELISA validation was performed following the current EMEA guidelines on bioanalytical method validation (EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2** Committee for Medicinal Products for Human Use, July 21, 2011. Link: https://www.ema.europa.eu/en/bioanalytical-method-validation). Accuracy and precision standards are commonly applied to ELISA assays. The following assay characteristics were tested to evaluate the assay performance and reliability of results: reference standards and linearity of the calibration curve within the concentration range, sensitivity indicated by the lower limit of quantification (LLoQ), reproducibility within an experiment and between experiments, matrix effect, dilution linearity, stability of the analyte over time and under different storage temperatures, selectivity of the binding reagents, specificity, clinical sample analysis, and cross-validation. Accuracy was determined as the percentage difference between measured and theoretical alemtuzumab concentrations. Precision was expressed as CV, which was defined as SD/mean value (%).

RESULTS

Reference Standards and Calibration Curve

Three different alemtuzumab calibration standards from 3 different batches of alemtuzumab (reference standards) were compared to exclude concentration differences between the batches. The concentrations of the standards in the 3 batches were calculated using a calibration standard prepared from batch 1. The accuracy ranged from 91% to 104%. The measurement of the lowest calibration standard containing 0.39 $ng \cdot mL^{-1}$ was imprecise, and this corresponded with the defined LLoQ as described below. The results are reported in the **Supplemental Digital Content** (see **Table S1**, http://links.lww.com/TDM/A610).

A complete calibration curve was measured 8 times on different days over a period of 1 year, and reproducibility was analyzed based on the precision (CV, %) and accuracy (%) of the measured values in comparison with the alemtuzumab theoretical concentration. For 6 of 7 calibration standards with theoretical concentrations of 0.78–25 $ng \cdot mL^{-1}$, the overall CV was between 2% and 11% and the overall accuracy was 95%–105%. The accuracy and precision of the lowest standards were 82% and 44%, respectively.

Because 86% of the calibration standards showed precision and accuracy within ±20% of the theoretical concentration (Table 1), the linearity of the relationship between theoretical concentration and observed concentration was accepted and the assay dynamic range was defined as ranging from 0.78 to 25 $ng \cdot mL^{-1}$.

A representative calibration curve of alemtuzumab is shown in Figs. 1A, B.

LLoQ

LLoQ of an ELISA assay is defined as the lowest analyte amount that can be determined with acceptable precision (CV $<$ 25%) and accuracy (75%–125%). The LLoQ of our ELISA method was determined by testing samples containing a range of alemtuzumab concentrations between 0.5 and 3.0 $ng \cdot mL^{-1}$ prediluted in 1% PBS/HSA for 7–8 times. The CV of the measured concentrations ranged from 5% to 16% and the accuracy from 94% to 122%. All concentrations were reliably quantifiable according to the EMA guidelines; thus, the LLoQ was set to 0.5 $ng \cdot mL^{-1}$. Notably, at a concentration of 1.5 $ng \cdot mL^{-1}$, an increase in the measured accuracy error (+22%) and CV (16%) of unknown origin was observed. The results are presented in the **Supplemental Digital Content** (see **Table S2** and **Figure S1**, http://links.lww.com/TDM/A610).

Reproducibility

For the assessment of the reproducibility of given ELISA assay, both accuracy and precision within an experiment (within-run) and between runs were tested.

TABLE 1. Calibration Curve

Alemtuzumab theoretical concentration ($ng \cdot mL^{-1}$)	25.00	12.50	6.25	3.13	1.56	0.78	0.39
Sample 1 (batch 1)	25.09	12.41	6.33	3.22	1.40	0.77	BQL
Sample 2 (batch 1)	25.02	12.38	6.27	3.41	1.60	0.67	0.12
Sample 3 (batch 1)	24.85	12.85	5.78	3.32	1.99	0.67	BQL
Sample 4 (batch 1)	25.00	12.51	6.21	3.18	1.58	0.70	0.43
Sample 5 (batch 1)	24.95	12.61	6.10	3.23	1.61	0.77	BQL
Sample 6 (batch 1)	24.45	13.00	6.00	3.19	1.75	0.90	BQL
Sample 7 (batch 1)	28.43	12.71	5.99	2.97	1.44	0.73	0.41
Sample 8 (batch 1)	24.84	12.65	6.20	3.05	1.72	0.75	0.31
N	8	8	8	8	8	8	4
SD	1.27	0.21	0.18	0.14	0.19	0.07	0.14
Average results	25.33	12.64	6.11	3.20	1.64	0.74	0.32
Average accuracy (%)	101	101	98	102	105	95	82
Average CV (%)	5	2	3	4	11	10	*44*

BQL, below the quantifiable limit.

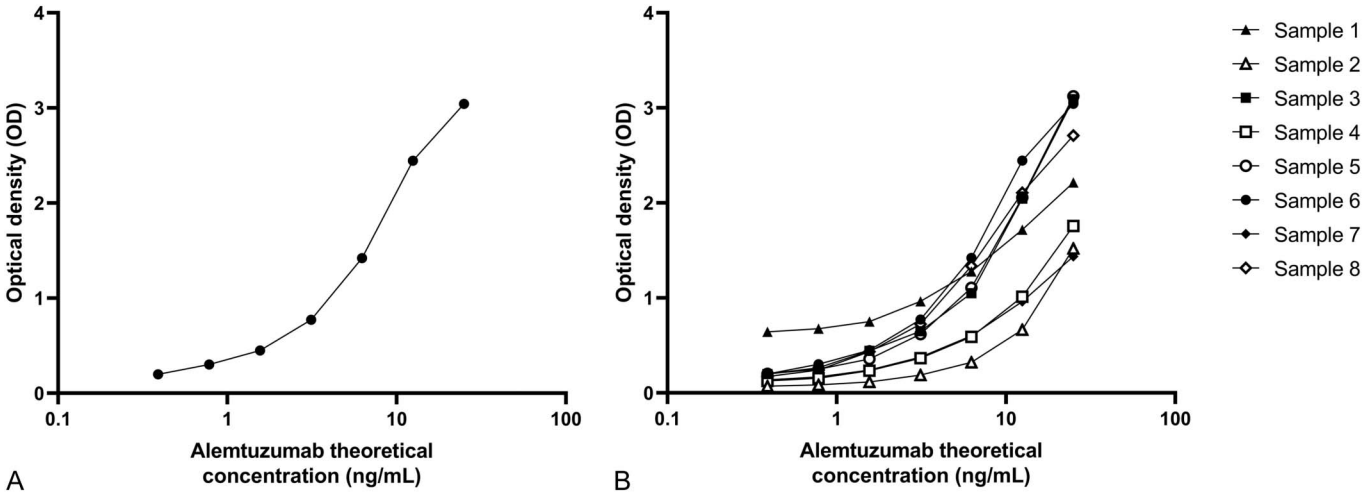

FIGURE 1. Calibration curve. (A) Representative calibration curve (sample 6 in Table 1). (B) Calibration curves for samples 1–8.

QC samples at 4 alemtuzumab concentrations (20, 12, 3, and 0.8 ng·mL^{-1}) were included in 6-fold in one assay. In the within-run assessment, overall accuracy was between 96% and 105% and overall CV was between 3% and 9% (Table 2 and Fig. 2A). The between-run assessment provided an overall accuracy between 86% and 95% and an overall CV between 5% and 14% (Table 3 and Fig. 2B). The reproducibility of this assay was proven in accordance with the EMA guidelines.

Matrix Effect

Pooled human serum and plasma samples were used to analyze whether matrix characteristics interfered with the quantification of alemtuzumab. The use of pooled serum and plasma was preferred over single serum/plasma donors because it causes potentially interfering conditions, such as hemolysis or high protein concentrations, better than individual samples. Extreme matrix conditions were determined by analyzing low (3 ng·mL^{-1}) and high (12 ng·mL^{-1}) alemtuzumab concentrations in triplicate in various concentrations of pooled human serum or plasma diluted in 1% PBS/HSA (90%–0.2%). No significant interference was observed for either matrix as accuracy was within 80%–120% in 17 of 20 (85%) samples, and CV was below 12% in all samples **Supplemental Digital Content** (see **Table S3**, http://links.lww.com/TDM/A610).

Dilutional Linearity

Because the dynamic range of the alemtuzumab ELISA is limited, we tested whether dilution of samples with concentrations of alemtuzumab exceeding the upper limit of reliable quantification resulted in the accurate determination of analyte concentrations (dilution linearity). Alemtuzumab from the vial containing 30 mg·mL^{-1} was diluted 1.2–38.4 × 10^6 times in 1% PBS/HSA and measured in 5-fold. Although the accuracy at each dilution was between 80% and 120% regardless of the dilution factor, the CV of the measured concentrations ranged from 3% to 11%, thus fulfilling the requirements of the EMA guidelines and excluding interference of dilution on alemtuzumab concentrations **Supplemental Digital Content** (see **Table S4**, http://links.lww.com/TDM/A610)

Stability Over Time and Under Different Storage Temperatures

Serum samples from children undergoing alemtuzumab treatment were routinely stored at −80°C in our laboratory and thawed when used for alemtuzumab quantification. For each stability assessment, in 1% PBS/HSA 3 separately prediluted samples with low (3 ng·mL^{-1}) and high (12 ng·mL^{-1}) alemtuzumab concentrations were prepared and tested in duplicate.

To assess the stability of alemtuzumab over time, alemtuzumab samples were stored without interruption at room temperature, 4, −20, and −80°C for 0 (samples were immediately measured), 4, 13, and 32 days before measurement. Except for the low alemtuzumab concentration after 32 days storage at 4°C, which presented a CV of 30%, the alemtuzumab concentration of all measured samples was measured with an accuracy of 84%–116% and a precision ranging from

TABLE 2. Reproducibility Within-Run Accuracy (%) and Precision (CV%)

Alemtuzumab theoretical concentration (ng·mL^{-1})	20.00	12.00	3.00	0.80
Sample 1	18.65	12.35	2.85	0.74
Sample 2	19.23	12.21	2.94	0.83
Sample 3	18.43	11.83	2.83	0.80
Sample 4	17.53	13.00	2.85	0.68
Sample 5	20.47	13.00	3.09	0.75
Sample 6	22.43	13.38	2.99	0.80
n	6	6	6	6
SD	1.75	0.59	0.10	0.05
Average results	19.46	12.63	2.93	0.77
Average accuracy (%)	97	105	98	96
Average CV (%)	9	5	3	7

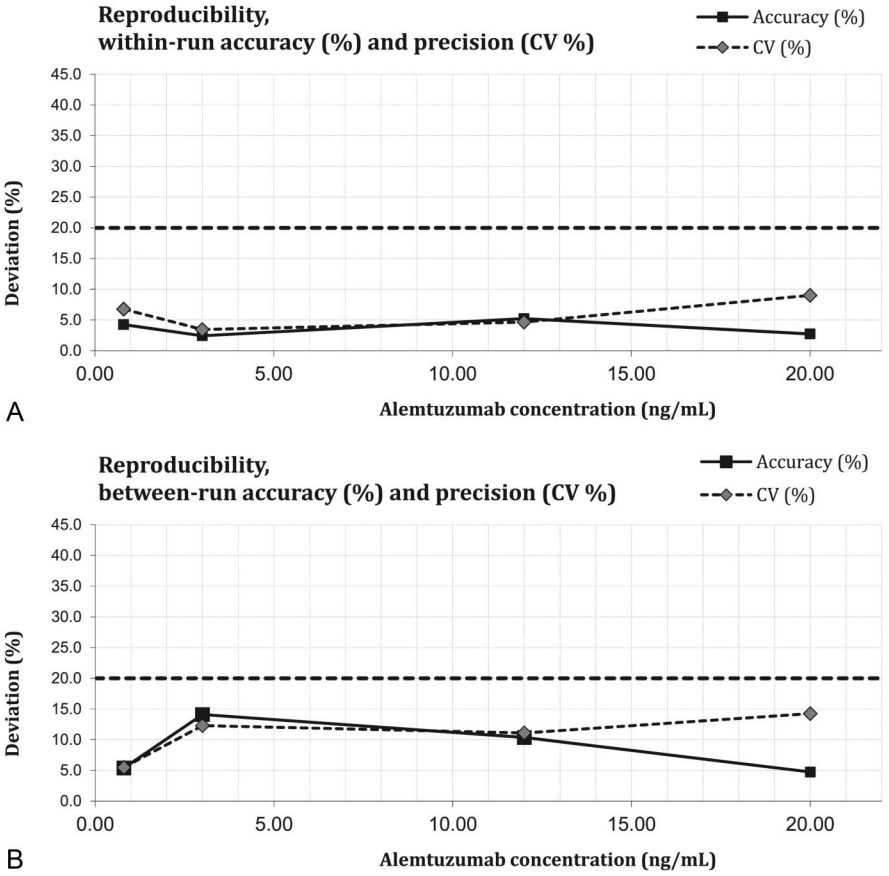

FIGURE 2. Reproducibility. (A) Within-run reproducibility with accuracy (%) and precision (CV %). (B) Between-run reproducibility with accuracy (%) and precision (CV %).

0% to 14% at each measured time point and storage temperature **Supplemental Digital Content** (see **Table S5**, http://links.lww.com/TDM/A610).

For stability assessment after multiple freeze–thaw (F–T) cycles, prediluted samples with low (3 ng·mL^{-1}) and high (12 ng·mL^{-1}) concentrations were subjected to 0–6 cycles of freezing at −80°C for at least 12 hours, thawed at room temperature for at least 1 hour and subsequently analyzed. The results demonstrated stable alemtuzumab concentrations after as many as 6 freeze–thaw cycles, with accuracy values ranging from 90% to 102% and precision levels of 2%–15%, thus fulfilling the requirements for acceptance in accordance with the EMA guidelines **Supplemental Digital Content** (see **Table S6**, http://links.lww.com/TDM/A610).

Selectivity and Specificity of the Binding Reagents

Children undergoing HSCT may receive other antilymphocyte antibodies before, during, or after alemtuzumab treatment. To test for analytical interference, we performed alemtuzumab quantification at 2 different theoretical concentrations (5 and 15 ng·mL^{-1}) in pooled human serum alone and in the concomitant presence of high concentrations (100 mcg·mL^{-1}) of 2 different polyclonal rabbit antibodies targeting T cells (antithymocyte globulin, ATG; Sanofi Genzyme; anti-T-lymphocyte globulin, ATLG; Neovii, Rapperswil, Switzerland) and rituximab (200 mcg·mL^{-1}, Roche, Basel, Switzerland), a humanized anti-CD20 IgG1 antibody.

The average level of accuracy was 98%–113% at higher and 102%–114% at lower concentrations **Supplemental Digital Content** (see **Table S7**, http://links.lww.com/TDM/A610). In accordance with the EMA guidelines, we concluded that no significant interference of the analyzed concurrent antibodies was observed at 2 different alemtuzumab concentrations (5 and 15 ng·mL^{-1}).

TABLE 3. Between-Run Accuracy (%) and Precision (CV%)

Alemtuzumab theoretical concentration (ng·mL^{-1})	20.00	12.00	3.00	0.80
Sample 1	23.00	11.80	2.50	NA
Sample 2	17.90	10.50	2.20	NA
Sample 3	17.79	9.69	2.22	0.81
Sample 4	19.50	12.60	2.90	0.77
Sample 5	20.90	10.30	2.80	0.71
Sample 6	15.22	9.65	2.84	0.74
n	**6**	**6**	**6**	**4**
SD	**2.71**	**1.19**	**0.32**	**0.04**
Average results	**19.05**	**10.76**	**2.58**	**0.76**
Average accuracy (%)	**95**	**90**	**86**	**95**
Average CV (%)	**14**	**11**	**12**	**5**

To assess the selectivity of the assay for alemtuzumab in the presence of other relevant analytes (specificity), we also measured samples containing no alemtuzumab but only high concentrations of rituximab (200 mcg·mL^{-1}), ATG (100 mcg·mL^{-1}), or ATLG (100 mcg·mL^{-1}). These samples provided no signal (the ODs were equal to the background), indicating that the assay reagents did not react with other therapeutic antibodies (data not shown).

Clinical Sample Analysis and Parallelism

To test the applicability of the ELISA in the clinical setting, 33 frozen samples of patient serum were tested in 1-fold to 3-fold to test the reproducibility and serially diluted 3 times in the plate to obtain 4 measurable dilutions per sample to detect possible differing affinities for alemtuzumab due to a matrix effect (parallelism). Sera were collected from children recruited for a PK/PD study (ARTIC study, NL8185/BASEC-2018-00794) on alemtuzumab before HSCT. High-concentration samples, as well as samples with lower drug concentrations, provided excellent reproducibility (within-run and between-run CV $<$ 30% in all but 1 sample with very low drug concentration), and the presence of a parallelism effect was excluded.

The PK analysis of the 2 representative patients with varying maximum concentrations and rates of clearance is shown in Fig. 3. Additional data are available in the **Supplemental Digital Content** (see **Table S7**, http://links.lww.com/TDM/A610).

Cross-Validation

To compare our ELISA with a commercially available ready-made recombinant, anti-idiotypic antibodies targeting alemtuzumab (HCA198, clone AbD19189, 0.5 mg·mL^{-1}; HCA175P HRP-labeled, clone AbD16942_hIgG1, 0.1 mg·mL^{-1}; Bio-Rad Laboratories, Hercules, CA) in 37 patient samples with a wide range of concentrations collected randomly from the same PK/PD study as described above (ARTIC study) were measured pair-wise in 2 runs using the same calibration standards and set of QCs. The agreement between methods was tested computing a Bland–Altman plot, which showed a mean difference between methods of 0.92% with a 95% interval of agreement ranging from −24.77% to 22.94% **Supplemental Digital Content** (see **Figure S2**, http://links.lww.com/TDM/A610). Passing and Bablok regression analyses were used for the slope and intercept computations **Supplemental Digital Content** (see **Figure S3**, http://links.lww.com/TDM/A610). Although the intercept was estimated at 0.00701 (bootstrap 95% confidence interval [CI] based on 999 bootstrap samples: −0.02156 to 0.03870), the estimated slope was 0.97927 (bootstrap 95% CI: 0.87483 to 1.05961).

DISCUSSION

The authors reported a validated, highly sensitive, accurate, and specific ELISA method to measure alemtuzumab levels in serum and plasma samples from children undergoing HSCT. Our "NC ELISA" assay was shown to be highly sensitive with an LLoQ of 0.5 ng·mL^{-1}, which was 200-fold lower than the concentration considered to be the threshold for lympholytic activity (0.1 mcg·mL^{-1}). The dynamic range of 0.78–25 ng·mL^{-1} was tested in a pediatric cohort treated with low-dose regimens intravenously (0.5–1.0 mg kg^{-1} iv), according to current clinical practice,[9,13] and perfectly comprised the measured alemtuzumab concentrations. Both the interassay and intra-assay accuracy and precision levels demonstrated the high reliability and reproducibility of this technique, which also provided high selectivity for the binding reagents. The missing cross-validation of our assay with another laboratory represents a limitation of this validation procedure because a different sample preparation may lead to a different result. However, satisfactory agreement of methods was shown in the cross-validation testing of our NC ELISA assay with a further ELISA method based on commercially available recombinant, anti-idiotypic antibodies (HCA antibodies).

Despite the common use of alemtuzumab in children undergoing HSCT, little is known regarding the PK and PD characteristics of this patient group. High interpatient alemtuzumab PK variability has previously been described in adult cohorts[12,14] and in a recent pediatric multicenter study by our group.[9] Accurate alemtuzumab quantification is crucial for PK and exposure–response studies in this patient population and may lead to the identification of determinants of variability, thereby allowing for optimization of alemtuzumab treatment in clinical practice. With a turnaround time of 6–8 hours and a standard alemtuzumab dosing interval of 24 hours, this ELISA is well suited for real-time PK monitoring.

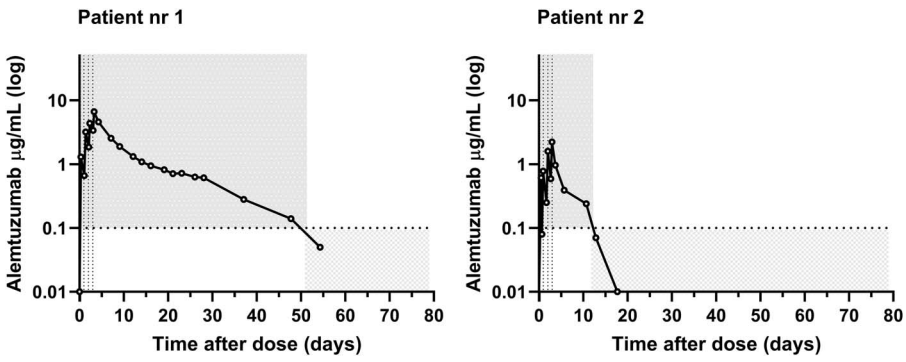

FIGURE 3. Clinical sample analysis. Alemtuzumab PK analysis in 2 representative patients. Dotted vertical lines indicate time points of alemtuzumab administration. Shaded areas represent the period above (dark gray) and below (light gray) the drug lympholytic level of 0.1 mcg·mL^{-1}.

Given its high sensitivity and reliability, this ELISA assay may be useful for developing pharmacological models that allow model-informed precision dosing based on limited sampling protocols combined with Bayesian estimation of alemtuzumab concentrations, as reported by Dong et al.[15]

Alemtuzumab measurements have been performed previously, but the reported methods have important limitations. Flow cytometry methods to measure alemtuzumab in serum, using a human T-cell line-expressing CD52 to capture alemtuzumab and anti-human IgG (Fc) FITC-labeled antibodies to detect bound alemtuzumab, have been reported to have an LLoQ of 0.1 and 0.25 mcg·mL^{-1}, respectively.[9,16] However, a sensitivity able to detect also concentrations below the drug lympholytic level of 0.1 mcg·mL^{-1} is required for reliable PK and exposure–response analysis.[1,11,12] The first sandwich ELISA for alemtuzumab quantification in adult patients with chronic lymphoblastic leukemia, which was based on monoclonal rabbit anti-rat IgG (absorbed against human IgG) to capture alemtuzumab, has been reported to have an LLoQ of 0.01 mcg·mL^{-1}.[17] Another ELISA based on locally produced rabbit polyclonal antibodies against the Fab fragments of alemtuzumab (containing rat sequences) was published subsequently.[18] The reported LLoQ was excellent, reaching 0.047 ng·mL^{-1}, but the lack of availability of the reagents remained a major limitation of this method. Preliminary experiments in our group testing commercially available recombinant anti-idiotypic antibodies targeting alemtuzumab were discouraged because of inconsistent performance (data not shown). An electrochemiluminescence bridging immunoassay provided an LLoQ of 60 ng·mL^{-} which is promising but still close to the drug lympholytic level (0.1 mcg·mL^{-1}) and significantly lower than the one reached with an ELISA technique.[19]

Another important advantage of the reported ELISA is the very limited volume of serum or plasma (~10 μL, which corresponds to ~20 μL of whole blood) required for each analysis, which may enable capillary blood sampling. Although current pediatric transplant protocols require a central venous line, and sampling for alemtuzumab PK analysis is combined with routine blood analysis, capillary sampling might be very useful in a different clinical context.

To the best of our knowledge, no data on capillary alemtuzumab measurements have been reported to date. However, considering the minimal amount of serum or plasma required for alemtuzumab quantification and the encouraging results provided by the measurement of other therapeutic monoclonal antibodies, such as infliximab[20] or adalimumab,[21] in patients with chronic inflammatory bowel diseases, capillary blood sampling, which can be performed at home, may provide a valid alternative to venous blood collection for alemtuzumab PK monitoring. In particular, adult patients treated with alemtuzumab over a long period for severe autoimmune diseases, such as MS, may benefit from capillary PK analysis for therapeutic drug monitoring. To the best of our knowledge, current MS trials are monitoring the response to alemtuzumab clinically and not according to PK data; however, this may be of interest for future studies. Further research is needed to investigate the stability of alemtuzumab after sampling using filter paper (dried blood spots, Hemaxis[22]) or innovative microsamplers, such as Mitra with volumetric absorptive microsampler technology,[23,24] and after the drying and subsequent elution processes.

CONCLUSIONS

Here, we report the development and validation of an efficient and highly sensitive ELISA method based on human polyclonal anti-idiotypic antibodies to quantify alemtuzumab concentrations in serum or plasma samples. This method can be used to develop population PK models and conduct exposure–response analyses to further optimize the use of alemtuzumab in pediatric patients undergoing HSCT.

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