

Evaluation of a Nanoparticle-Based Busulfan Immunoassay for Rapid Analysis on Routine Clinical Analyzers

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Background: Busulfan is an alkylating agent used in allogeneic hematopoietic stem cell transplantation for various malignant and nonmalignant disorders. Therapeutic drug monitoring of busulfan is common because busulfan exposure has been linked to veno-occlusive disease, disease relapse, and failed engraftment. The authors developed an automated immunoassay, along with stable calibrators and controls, and quantified busulfan in sodium heparin plasma.

Methods: The authors evaluated a homogenous nanoparticle immunoassay, the MyCare Oncology Busulfan Assay Kit (Saladax Biomedical, Inc), for precision, sensitivity, accuracy, and linearity on an open channel clinical chemistry analyzer; they compared the method with 2 mass spectrometry methods (liquid chromatography–tandem mass spectrometry and gas chromatography/mass spectrometry), using anonymized, remnant patient samples.

Results: The coefficients of variation for repeatability and within-laboratory precision were $\leq 9.0\%$. The linear range was 150–2000 ng/mL; samples up to 6000 ng/mL can be measured with sample dilution. Measured values deviated by $\leq 14\%$ from assigned values. Comparison between validated mass spectrometry methods resulted in a correlation coefficient $R \geq 0.995$.

Conclusions: The MyCare Busulfan Assay Kit shows the precision, accuracy, linearity, and test range for performing busulfan

concentration measurements in sodium heparin plasma on routine clinical chemistry analyzers.

Key Words: immunoassay, busulfan, therapeutic drug monitoring, hematopoietic stem cell transplantation

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INTRODUCTION

Busulfan is a widely used bifunctional alkylating agent in allogeneic hematopoietic stem cell transplantation for various malignant and nonmalignant disorders. Originally, one of its primary uses was conditioning regimens for treating patients with chronic myelogenous leukemia, but its use for the treatment of this disease has decreased considerably because of newer targeted tyrosine kinase inhibitors. Recently, the use of preparative regimes with busulfan for bone marrow transplantation has increased for both cellular and gene therapy.

The pharmacokinetic variability of busulfan has been characterized for more than 25 years, such as its narrow therapeutic range and pharmacodynamic associations related to its exposure.^{1–4} Overexposure has been associated with an increased risk for toxicities, such as veno-occlusive disease, mucositis, and transplantation-related mortality. Underexposure has been associated with a higher risk of graft rejection or disease relapse. The package insert of Busulfex (IV busulfan) recommends specific exposure targets in pediatric patients.⁵ To ensure that busulfan exposure is within the therapeutic range, personalized busulfan dosing through therapeutic drug monitoring (TDM) is common. Personalized dosing of busulfan to achieve therapeutic exposure has lowered the risk of disease relapse⁴ and lowered the incidence of veno-occlusive disease.¹

The TDM of busulfan is performed with physical methods, such as high-performance liquid chromatography–ultraviolet and various mass spectroscopic (MS) methods.^{6–10} These methods are performed at a limited number of centers, necessitating the shipment of samples from the transplantation center to an outside laboratory. The turnaround time can be problematic because the results may not be available before the next dose is administered, delaying dose adjustment and optimization of exposure. In addition, the instability of busulfan creates challenges in routine laboratory operations and single-use frozen calibrators may lead to interassay imprecision. A stabilized liquid calibrator, which is stable for at least 1 year at 4°C, will be easier to use in the laboratory.

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The objective of this study was to develop a rapid and quantitative assay for busulfan that would facilitate on-site testing of busulfan possible at any clinical chemistry laboratory. Novel antibodies were developed and applied to a nanoparticle immunoassay format.¹¹ Nanoparticle immunoassay affords advantages: (1) high sensitivity to its target analyte; (2) reagent stability (ie, greater than 1 year); and (3) instrument flexibility.

MATERIALS AND METHODS

Principle of the Immunoassay

The immunoassay is based on changes in the scattering of light that occurs when nanoparticles aggregate. The immunoassay is composed of 2 reagents: reagent 1 (R1), a buffer solution containing a multivalent busulfan conjugate, and reagent 2 (R2), a solution of nanoparticles that are coated with monoclonal antibodies selective for busulfan.¹¹ When the 2 reagents are mixed in the analyzer, the nanoparticles aggregate, as the antibodies on the particles bind to the multivalent conjugate. The aggregated particles cause the incident light to scatter, leading to a shift in absorption that is measured by the analyzer. Busulfan in the sample and the busulfan conjugate compete to bind to the antibodies on the nanoparticles. Aggregation of the nanoparticles is inhibited as busulfan binds to the antibody and prevents binding to the busulfan conjugate, leading to less light scattering and a lower absorbance. Thus, the aggregation of the particles and absorbance is dependent on the concentration of busulfan in the sample. An inhibition curve is obtained, with the maximum absorbance occurring at low drug concentration, and the absorbance decreases as the busulfan concentration increases. This curve is generated by fitting the absorbance versus busulfan concentration data to a 4-parameter regression equation (logit/log4).

Assay Method

Evaluation of the busulfan immunoassay was conducted at Saladax Biomedical (Bethlehem, PA) on 2 Beckman Coulter AU480 analyzers (Brea, CA).

The MyCare Oncology Busulfan Assay Kits for reagents, calibrators, and controls were obtained from Saladax Biomedical (Bethlehem, PA). The kits contained 2 liquid reagents (reagent 1 and reagent 2, described above), 6 calibrators, and 3 controls. Both R1 and R2 were supplied in bottles compatible with the Beckman Coulter analyzers (Brea, CA). The assay calibrators and controls, consisting of a stable busulfan analog in plasma, were supplied in dropper bottles at levels 0, 150, 300, 600, 1200, and 2000 ng/mL and 225 (low), 450 (medium), and 900 ng/mL (high), respectively.

The AU480 is an open channel clinical chemistry analyzer; 95 μ L of R1 and 10 μ L of sample were mixed in the reaction quartz cuvette. After a short incubation period (3.5 minutes), 95 μ L of R2 was added and mixed together. The end point signal (ie, the optical density of the reaction) was calculated at 600 nm from the difference in the reaction at time point 12 (3.8 minutes after sample addition) and at time

point 27 (8.6 minutes after sample addition). A calibration curve was generated for busulfan concentrations 0–2000 ng/mL, fitted to a logit/log4 regression model.

Calibration and Standardization

Busulfan is unstable at ambient room temperature and is stable for 24 hours when stored at 4°C.¹² Thus, using busulfan as a calibrator in routine clinical laboratory analysis would be challenging because single-use frozen calibrators and controls would be required, adding to interlaboratory imprecision. The structures of busulfan and the stabilized busulfan analog are shown in Figure 1; the substitution of nitrogen for oxygen atoms in the labile methane sulfonate groups creates a stabilized standard.^{11,13}

The difference in antibody binding to busulfan and the stabilized busulfan analog was used to assign apparent busulfan concentrations to the calibrators and controls. The cross-reactivity of the busulfan analog in the immunoassay was determined by calibrating the immunoassay with plasma spiked with United States Pharmacopeia-certified busulfan, concentrations of which were independently verified by mass spectrometry and measuring the stabilized busulfan analog calibrators. Calibrator assignment values were confirmed by verifying assay linearity using independent busulfan-spiked plasma samples, as described in the testing procedures further.

Stability of the stable busulfan analog in the plasma calibrators and controls was evaluated at 4 temperatures: 4, 25, 37, and 45°C. After 12 weeks (4 and 25°C), 21 days (37°C), or 9 days (45°C), the calibrators and controls were measured by conducting the immunoassay. The calibrators and controls were considered stable if their assay signal after storage deviated by $\leq 3\%$ of the assay signal on day 0.

Sample Preparation

Busulfan-spiked plasma (sodium heparin) samples were prepared from a stock solution of busulfan (Millipore Sigma, St. Louis, MO) in dimethyl sulfoxide. Samples were spiked such that the amount of dimethyl sulfoxide in each sample was $< 0.01\%$ wt/wt. The samples were prepared in bulk, aliquoted into 1.0 mL volumes, and stored at -80°C . On each testing day, aliquots were thawed at 4°C for at least 2 hours before use. Aliquots were not refrozen and were discarded after 12 hours at 4°C.

Testing Procedures

The repeatability and within-laboratory precision of the assay were evaluated using the 3 assay controls and 4 busulfan-spiked plasma pools at concentrations of 200, 600, 1000, and 1600 ng/mL. Each sample was measured on one analyzer in duplicate, twice a day for 5 days, for a total of 20 replicates per sample. The results were evaluated using the Complex Precision module in the EP Evaluator version 12 (build 12.1.0.18, Data Innovations, South Burlington, VT).

The linearity of the assay was evaluated using 10 busulfan-spiked plasma pools at nominal concentrations of 100, 200, 400, 600, 800, 1000, 1300, 1600, 1800, and 2100 ng/mL. Four replicates of each sample were measured on one analyzer. All testing occurred in one day. The results were

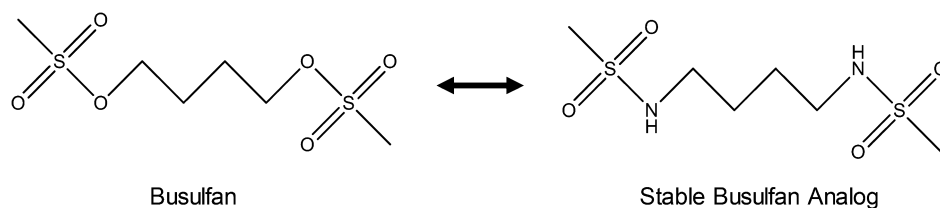


FIGURE 1. Structures of busulfan and stable busulfan analog.

analyzed using the EP6 Linearity module in EP Evaluator, version 12 (build 12.1.0.18, Data Innovations). The individual and mean recoveries of the linearity spikes were calculated to evaluate the accuracy of the assay. Dilution linearity was demonstrated using spiked busulfan plasma pools at concentrations of 2500, 3000, 4000, and 5000 ng/mL, either on-analyzer or off-analyzer, using deionized H₂O.

The limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ) of the assay were determined using spiked busulfan samples in plasma that was negative for busulfan from 3 donors. Each sample was measured in triplicate on 2 analyzers for 3 days, for a total of 36 replicates per sample. The LOB was defined as the 95th percentile value of the $n = 36$ data set of the 0 ng/mL busulfan sample. The LOD was defined as the median value observed at the lowest spiking concentration for which the $n = 36$ data set had $\leq 5\%$ of results below the LOB. The LOQ was defined as the mean value observed at the lowest spiking level for which the $n = 36$ data set had a total error of $\leq 35\%$ by the Westgard model, as described in the CLSI Guideline EP17-A2.¹⁴

Anonymized remnant heparin plasma samples were obtained from patients undergoing busulfan treatment from the Hospital of the University of Pennsylvania ($n = 132$) and the University of Texas MD Anderson Cancer Center ($n = 99$). The busulfan concentrations in these samples were determined using gas chromatography/mass spectrometry^{15,16} or liquid chromatography-tandem mass spectrometry (LC-MS/MS). After measurement, the samples were frozen and shipped to Saladax Biomedical on dry ice. On receipt, samples were stored at -80°C . On the day of testing, all samples were thawed at 4°C for at least 2 hours before measurement in the immunoassay. The immunoassay methods on the Beckman Coulter AU480 and mass spectrometry were compared. Results were evaluated using the Deming regression analysis. To ensure that the calibration curves between sites were comparable, spiked busulfan plasma samples were also measured by mass spectrometry. To account for calibrator differences between the immunoassay and one of the sites, the spiked busulfan results were used to normalize the immunoassay calibration curve to the LC-MS/MS calibrators.

RESULTS

Calibrator and Control Stability

The stability of the calibrators and controls at 4°C was predicted by measuring the stability at 25, 37, and 45°C . After storage at these elevated temperatures, there was $\pm 2\%$ deviation in the optical density values, as shown in **Supplemental**

Digital Content 1 (see **Table S1**, <http://links.lww.com/TDM/A489>), indicating that the busulfan analog in the calibrators was stable. The Q10 rule (factor 3) predicts 2 years of stability at 4°C , as shown in the **Supplemental Digital Content 2** (see **Information**, <http://links.lww.com/TDM/A489>).

Calibration Curve

The calibration curve was based on 6 busulfan concentrations between 0 and 2000 ng/mL, with a nonlinear curve fit (logit/log4). The measuring range can be extended to 6000 ng/mL with an automatic dilution factor of 3. The total span of the assay was approximately 230 milli-absorbance units (mA), with calibrator A (0 ng/mL) starting at 375 mA and calibrator F (2000 ng/mL) ending at 145 mA. The calibration curve was stable for at least 14 days, where stability was assessed by control values within the specification without having to recalibrate, as shown in the **Supplemental Digital Content 3** (see **Table S2**, <http://links.lww.com/TDM/A489>).

PRECISION

The repeatability and within-laboratory coefficient of variations (CVs) of the 3 assay controls (225, 450, and 900 ng/mL busulfan) and 4 spiked busulfan plasma samples (200, 600, 1000, and 1600 ng/mL) were calculated and are shown for each sample in Figure 2. The CVs were $\leq 5.8\%$ for all samples, except for the lowest spike sample of 200 ng/mL, where the CVs were 6.1% and 9.0% for repeatability and within-laboratory precision, respectively.

SENSITIVITY

The LOB was 17 ng/mL based on measurements of 3 donor plasmas negative for busulfan, whereas the LOD and LOQ were 109 ng/mL and 150 ng/mL, respectively.

Linearity and Accuracy

Linearity was considered acceptable if nonlinearity was $\leq 15\%$ using EP evaluator. The assay was linear over an assay range of 150–2000 ng/mL (Fig. 3). The results yielded a slope of 0.98, an intercept of -18 , and a correlation coefficient (R) of 0.9998. The mean recovery of the samples in the given range was 86%–98%, and the individual recovery was 82%–100%. The mean recovery of samples diluted with water at a dilution factor of 3 was 96%–102% (see **Table S3**, **Supplemental Digital Content 4**, <http://links.lww.com/TDM/A489>). Therefore, the clinical reportable range with dilution was 150–6000 ng/mL.

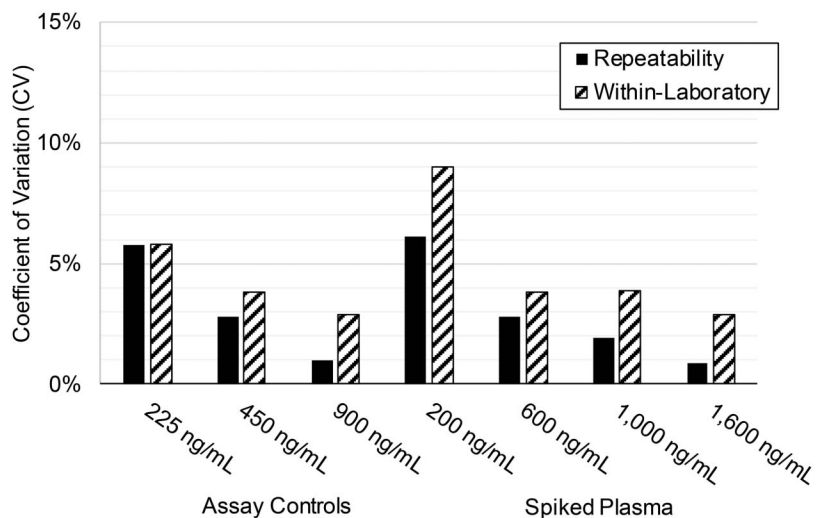


FIGURE 2. Repeatability and within-laboratory CV of plasma controls and spiked plasma samples in busulfan immunoassay.

Method Comparison

Deidentified patient samples from the Hospital at the University of Pennsylvania were measured on one analyzer, whereas those from the University of Texas MD Anderson Cancer Center were measured on 2 analyzers, using the busulfan immunoassay.

Regression analysis between the immunoassay and mass spectrometry was performed separately for each mass spectrometry method. Deming regression statistics between the immunoassay and laboratory 1 were a slope of 0.96, a y-intercept of 10, and a correlation factor (R) of 0.997. Deming regression statistics between the immunoassay and laboratory 2 were a slope of 0.80, a y-intercept of 37, and an R of 0.999. For laboratory 2, although a high correlation to the immunoassay was observed, a slope of 0.80 indicated a difference in standardization. This difference was confirmed by measurement of spiked busulfan samples in the immunoassay and by the mass spectrometry method of laboratory 2, where the samples read 20% higher by mass spectrometry. The immunoassay results of

the clinical samples from laboratory 2 were recalculated based on this standardization difference.

Once restandardized, a Deming regression for all clinical samples between the immunoassay and the mass spectrometry methods (n = 231) was obtained (Fig. 4 and see **Fig. S1, Supplemental Digital Content 5**, <http://links.lww.com/TDM/A489>): a slope of 0.95, a y-intercept of 16, and an R of 0.993. The average percentage bias was -3.6%. In addition, a Deming regression between the immunoassay values on the 2 analyzers (n = 99) was obtained: a slope of 0.995, a y-intercept of 26, and an R of 0.999 (Fig. 5).

Interferences

The effects of endogenous interferents such as human serum albumin (HSA), human immunoglobulin G (hIgG), triglycerides (TRI), hemolysis (Hb), bilirubin (BIL), rheumatoid factor (Rh), uric acid (UA), and human anti-mouse antibodies (HAMA) in the immunoassay were evaluated using contrived samples. No significant assay bias was

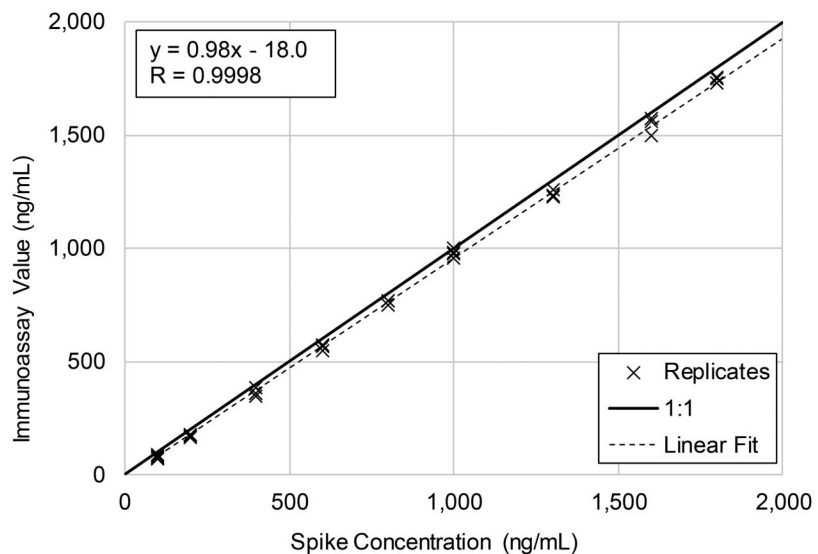


FIGURE 3. Linearity measurement of the immunoassay.

observed at clinically relevant concentrations (see **Table S4, Supplemental Digital Content 6**, <http://links.lww.com/TDM/A489>).

DISCUSSION

Good analytical performance of the immunoassay was demonstrated. In addition, the immunoassay correlated with mass spectrometry results obtained from the 2 laboratories for the deidentified samples from patients undergoing busulfan treatment. Minimal bias was also observed, indicating good agreement between the immunoassay and 2 mass spectrometry methods. Results for AU480 instruments were correlated, demonstrating the reproducibility of the assay using multiple instruments. The patient sample results also showed that there was no interference from endogenous substances in plasma or to busulfan metabolites, consistent with previous work with the busulfan antibody.¹¹ In addition, endogenous interference testing with contrived samples showed no significant assay bias in the presence of HSA, hIgG, TRI, Hb, BIL, Rh, UA, and HAMA.

A stable busulfan analog was used to prepare assay calibrators and controls, which affords stability at 4°C and ambient room temperature. As shown in Figure 1, the bridging oxygens in busulfan were isoelectrically substituted for nitrogen to create a stable busulfan analog. The labile methane sulfonate groups give busulfan its alkylating ability and are the cause of the instability of busulfan in plasma at 4°C and ambient room temperature. This instability makes busulfan a poor choice as a calibrator for a clinical chemistry assay because single-use frozen calibrators and controls are required to ensure the integrity of the calibrators. The sulfonamide replacement chemically stabilizes the molecule because they are less reactive than the methane sulfonate groups.

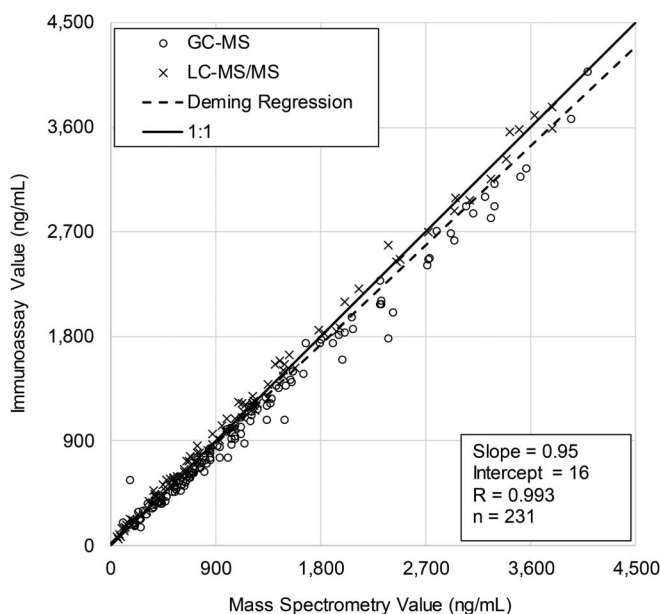


FIGURE 4. Comparison of immunoassay results to GC-MS and LC-MS/MS results.

Accelerated stability studies at 25°C, 37°C, and 45°C predicted that the stable busulfan analog plasma calibrators and controls would be stable for 2 years at 4°C using the Q10 rule (factor 3). Real-time stability of the calibrators and controls was at least 12 weeks at 4°C and 25°C. Liquid calibrators and controls provide a convenient alternative to frozen calibrators.

A homogenous nanoparticle immunoassay, with liquid calibrators and controls, was developed for the quantification of busulfan for use in automated clinical chemistry analyzers. Although the busulfan immunoassay was developed on the Beckman Coulter AU480 analyzer, the reagents for the immunoassay are not analyzer specific and can be used on other clinical chemistry analyzers with open channels. As clinical chemistry analyzers are available at most hospitals, this immunoassay is a suitable alternative to shipping samples to an external laboratory, thus providing busulfan concentration results in a clinically relevant time frame before the next dose. The liquid-stabilized calibrators and controls are easier to use in routine laboratory operations. Busulfan is dosed for 4 days (usually once daily, much less frequently 4 times per day), and an offsite laboratory requires 24–36 hours to return a result, by which time 25%–50% of the total dose will have already been administered, thus hindering any change to the dosage. A shorter turnaround time results in a much sooner dose adjustment to achieve the target busulfan exposure, leading to faster intervention to prevent busulfan overexposure or underexposure.

CONCLUSIONS

A homogenous nanoparticle immunoassay for the quantification of busulfan, using stable calibrators, will enhance widespread patient busulfan testing in clinical laboratory settings. The ease of use, lower cost, and faster

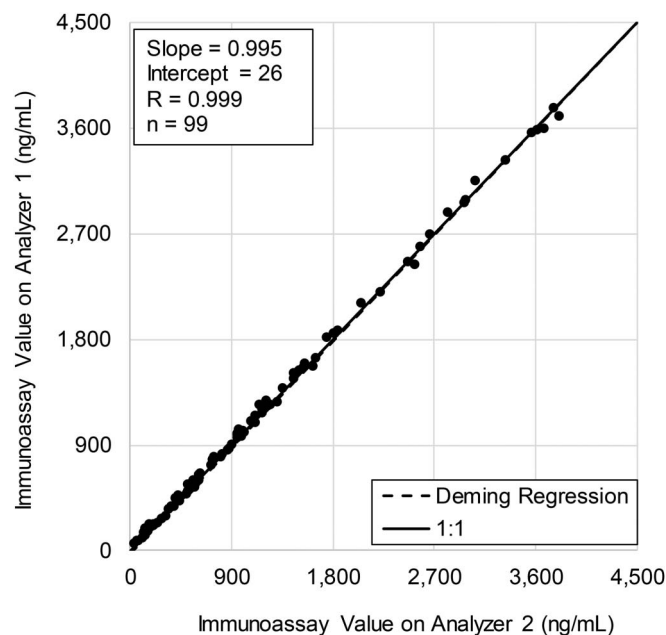


FIGURE 5. Comparison of analyzer-to-analyzer immunoassay results.

turnaround time enable busulfan TDM to be more readily available to optimize outcomes with hematopoietic stem cell transplantation conditioning regimens.

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