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Therapeutic Drug Monitoring of Busulfan for the Management of Pediatric Patients: Cross-Validation of Methods and Long-Term Performance

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Background: Busulfan (Bu) is an alkylating agent used as part of the conditioning regimen in pediatric patients before hematopoietic stem cell transplantation. Despite intravenous (IV) administration and dosing recommendations based on age and weight, reports have revealed interindividual variability in Bu pharmacokinetics and the outcomes of hematopoietic stem cell transplantation. In this context, adjusting doses to Bu's narrow therapeutic window is advised. We aimed to assess the utility of therapeutic drug monitoring (TDM) of Bu in children, the reliability of Bu quantification methods, and its stability in plasma when stored for up to 5 years.

Methods: Eighteen patients from our TDM center (252 samples) were included. All of them received a 2-hour Bu IV infusion 4 times daily for a total of 16 doses. The first dose of Bu was age/weight-based, and the subsequent doses were adjusted from third or fifth dose onward based on the estimated first dose pharmacokinetic parameters to target steady-state concentrations (Css) of 600–900 ng/mL. The performance of our unit's high-performance liquid chromatography with tandem mass spectrometry method was assessed using a quality control (QC, 35 series) chart. International, multicenter, cross-validation test (n = 21) was conducted to validate different analytical methods. To assess Bu stability, regression analyses and Bland–Altman plots were performed on measurements at repeated time points on samples stored at −80°C for up to 5 years.

Results: We observed a 4.2-fold interindividual variability in Bu Css after the first dose, with only 28% of children having a Css within the target range. During the 4 days of conditioning, 83% of children had their doses modified according to TDM recommendations. This achieved a Css within the target range in 75% of the children. Routine QC measurements were generally within the ±15% range around theoretical values, showing the optimal robustness of our center's analytical method. Two of the 21 Bu TDM centers returned inadequate results during cross-validation testing; both used a UV detection method. Storage at −80°C led to a fall in Bu content of 14.9% ± 13.4% at 2–4 years and of 20% ± 5% by 5 years ($r^2_{overall}$ = 0.92).

Conclusions: We conclude that TDM is an effective method of achieving targeted Bu levels in children. QC programs are crucial to monitoring and maintaining the quality of an analytical method.

Key Words: TDM, quality control, busulfan, children, stability

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INTRODUCTION

Busulfan (Bu) is a bifunctional alkylating agent widely used as a part of the conditioning regimen for children before hematopoietic stem cell transplantation (HSCT). Bu-based conditioning has been increasingly used in pediatric patients to prevent total body irradiation (TBI)–related side effects, including growth retardation and endocrine malfunction.[1,2] Wide interindividual variations in pharmacokinetics (PKs) and dynamics are observed with the classic weight/age-based intravenous (IV) Bu dose recommendations,[3–6] notably in children.[5,7–10] This PK variability is of concern as higher Bu plasma area under the curve (AUC) levels are associated with an increased risk of toxicity, including mucositis, hepatic sinusoidal obstruction syndrome (SOS), or acute graft versus host disease (aGvHD), which could ultimately be fatal.[11–13] If Bu AUCs are too low, graft rejection or relapse may occur.[12,14,15] Bu steady-state concentrations (Css) in plasma of 600–900 ng/mL,[14,16] 616–1026 ng/mL,[17] or more recently an AUC$_{cum}$ of 18,963–24,555 μmol·min/L (equivalent to a narrower Css of 811–1077 ng/mL) have been reported in

children with better outcomes.[12] Adjusting Bu doses to achieve concentrations within the therapeutic target range for different patients have been widely reported to improve clinical outcomes.[12,14,15] Several pediatric PK models exist for dose prediction;[10,18,19] yet information on optimal pediatric therapeutic windows is still scarce.

Therapeutic drug monitoring (TDM) involves measurement of the drug levels in plasma or blood and then individualization of drug dosage to achieve and maintain a drug concentration within a targeted therapeutic range. Bu TDM combined with model-based repeat-dose adjustment has been reported to be beneficial in children.[20,21] Several Bu quantification methods have been published using enzyme-linked immunosorbent assay,[22,23] liquid chromatography with UV detection,[24] gas chromatography with mass spectrometry,[25] and liquid chromatography with tandem mass spectrometry (LC–MS/MS),[26,27] all with varying plasma volumes, sensitivities, and process durations. Because the estimation of PK parameters requires collecting repeated blood samples, taking small volumes is highly recommended for pediatric patients.[28] Moreover, short turnaround times (sample reception to report dispatch to dose adjustment) are crucial for Bu, which is conventionally infused every 6 hours for 4 days. We have previously proposed an analytical method enabling rapid Bu dose adjustment and intermediate random verification of target levels.[28]

As patients' Bu plasma levels are the basis for each ensuing step in Bu dose personalization, assessment of accuracy of the quantification method is essential. Incorporating internal and external quality controls (QC) enables an assessment of the method's reliability. Within the context of a worldwide randomized pediatric study on TBI versus chemical agents, the centers dosing Bu participated in a cross-validation exercise for validating analytical methods for the quantification of Bu. When it comes to the stability issue, it has been shown that Bu in plasma is stable for at least 2 years.[28,29] However, information on the long-term stability of plasma of Bu patients is limited. The study of the rare diseases with low recruitment rate, such as the indications of Bu administration in children, could benefit from an increase in the number of eligible patients and the analysis of their stored samples, providing Bu is stable. This study assesses the quality of the analyses using (1) the variation of the QC samples incorporated into each routine Bu analysis over a period of 5 years, (2) the results of cross-validation in 21 Bu TDM centers around the world, and (3) Bu stability in plasma and dried plasma spots (DPSs) stored at −80°C up to 5 years. This study also aimed to evaluate the relevance of IV Bu TDM in a cohort from a pediatric Onco-Hematology Unit (Geneva University Hospitals, Switzerland). This was performed using the frequency of dose adjustments and investigating the association between Bu levels and toxicity, especially aGvHD.

MATERIAL AND METHODS

Pediatric Cohort

This observational study was approved by Geneva University Hospitals' Ethics Committee and recruitment occurred from December 2010 to December 2015 in the Pediatrics Department's Onco-Hematology Unit. Eighteen children scheduled for HSCT and about to begin an IV Bu-based conditioning regimen were enrolled after written informed consent from the patients and/or their legal representatives. A 2-hour IV Bu infusion was given 4 times a day for a total of 16 doses.[17] The first dose was age/weight-based, calculated according to hospital guidelines.[17] Briefly, dose ranges were age/weight-adjusted so that 0.8, 1.0, and 0.8 mg·kg^{-1} per dose were given to children aged from 3 to 12 months, 1–4 years, and above 4 years, respectively.[17] PK parameters of the first dose level were estimated at 6 time points (before initiation and at 120, 130, 150, 180, and 360 minutes after IV Bu initiation); Bu doses were then adjusted accordingly to hit a target range Css of 600–900 ng/mL.[17] The extent of dose modifications was evaluated using the ratio of the adjusted dose to the initial dose.

Additional TDM was performed at dose 3 (day 1) and then from dose 5 (day 2) to dose 9 (day 3), when guidelines suggest that verification of Bu exposure is required. Patients' demographic characteristics, medication, clinical outcomes, and side effects were extracted from their medical files.

Pharmacokinetic Parameters

Bu levels in plasma, as represented by the AUC, were estimated according to a non-compartmental model implemented using WinNonlin software (Pharsight Corp v3.1) ($AUC_{0-\infty}$ at first dose level or AUC_{0-last} at steady state). Subsequently, the estimation of PK parameters and dose adjustment recommendations were predicted using the Bayesian estimates of the NextDose calculator (www.nextdose. org).[30] The patient's cumulative Bu exposure, corresponding to the total administration (16 doses), was calculated by summing $AUC_{0-\infty}$ at the first dose and the estimated AUC_{0-last} of the following adjusted doses as follows. Cumulative AUC (AUC_{cum}) = (initial measured $AUC_{0-\infty}$) × (number of doses given before dose adjustment) + (adjusted daily AUC_{0-last}) × (number of doses given after the corresponding dose adjustment).

Clinical Outcomes and Diagnosis

The diagnosis of SOS was based on the modified Seattle criteria.[31] An aGvHD grade ≥2 was diagnosed and rated according to McDonald and the Consensus Conference on aGvHD.[32,33] Neutrophil recovery was defined as the first 3 consecutive days with an absolute neutrophil count ≥0.5 × 10^9 per liter. Graft failure or rejection was defined as persistent pancytopenia with no evidence of a hematologic recovery of donor cells beyond 28 days from the transplantation, or as a rapid decrease in neutrophil count and chimerism after successful engraftment.[34] Any relapse or rejection was recorded during the follow-up lasting at least 1 year.

Bu Quantification Method and Process Reliability

High-performance liquid chromatography–grade acetonitrile, methanol, and Bu were purchased from Sigma Aldrich (Buchs, Switzerland) and deuterated-Bu (d8-Bu) was

purchased from Toronto Research Chemicals (Toronto, Canada).

Bu TDM was performed using an in-house cross-validated LC–MS/MS method.[28] Briefly, 5 µL of EDTA-blood and/or plasma were spotted onto a Whatman 903 DBS card (GE Healthcare Europe GmbH, Switzerland). The Bu DPSs were extracted using methanol spiked with the d8-Bu internal standard and analyzed using LC–MS/MS (API 4000 triple quadrupole mass spectrometer; AB Sciex, Concord, Canada). Intra- and inter-day Bu coefficients of variation were <15%. Two independent Bu stock solutions in methanol were freshly prepared and serially diluted to obtain a calibration curve and a QC level, respectively.

Aliquots of plasma samples (from EDTA-blood) and spare DPS spots were immediately frozen at −80°C until repeat analyses. A control chart process was established with low (300 ng/mL), intermediate (600 ng/mL), and high (1400 ng/mL) Bu QC levels for the routine Bu analyses. These were analyzed at the beginning of the series and then randomly distributed during the analyses. QC samples from other laboratories and some cross-validation tests were often inserted in the series as well.

Cross-Validation

Allogeneic Stem Cell Transplantation for Children and Adolescents with Acute Lymphoblastic Leukemia (ALL SCTped 2012 FORUM, NCT01949129, NCT 02670564) is a worldwide, multicenter, randomized, controlled, prospective phase II/III study of the therapy and therapy optimization between TBI and chemotherapy for children and adolescents with ALL and undergoing allogenic HSCT. A vast worldwide cross-validation was performed involving all the TDM centers involved in the Bu arm of this study. Eight blinded QC samples were sent to each participating center (from October 2014 to June 2015, see Acknowledgments). The reported values were compared with theoretical ones as follows: Bias (%) = (Bu$_{reported}$ − Bu$_{theoretical}$)/Bu$_{theoretical}$. Criteria for acceptance were inspired by US Food and Drug Administration (FDA) and European Medicines Agency guidelines[35,36] as follows. Bias should be (1) within ±15% of the labeled concentration for at least one low (0–500 ng/mL), one intermediate (500–1500 ng/mL), and one high (1500–5000 ng/mL) Bu concentration and (2) a minimum of 5/8 QCs should be within those limits. To exclude any logistical problems (eg, QC shipment), a second QC set was sent to the laboratories that failed the first test. Finally, laboratories were considered to be not cross-validated when 2 consecutive tests provided unsatisfactory results.

Long-Term Bu Stability

The original (ie, at the start of the patients' conditioning regimen) and the repeat measurements were obtained using the same analytical method. Spearman rank correlation coefficients (r^2) were calculated for the original and repeat analyses and Bland–Altman method was used to calculate the mean difference between 2 measurements (bias) and the mean difference (1.96 SD).[37] Seventy-six samples obtained from 10 patients at our center, stored for a minimum of 2 years at −80°C, were included in this analysis.

Statistical Analyses

The correlations between Bu exposure and demographic characteristics such as age and weight were assessed using Spearman rank correlation (r^2). The associations between Bu exposure and treatment-related toxicity (aGvHD) were studied using the Student *t* test. Statistical analyses were performed using Stata 11.2 software (StataCorp LP, College Station, TX) and GraphPad Prism 5 (GraphPad Software, San Diego, CA). Mean and SD are presented unless otherwise specified. All tests were 2-sided, and a *P* value of ≤0.05 was considered statistically significant.

RESULTS

Pediatric cohort

Eighteen patients were included in this study. Patients' characteristics, Bu exposure, and cumulative incidences of outcomes are described in Table 1. More than one TDM measurement (i.e., measuring Bu plasma levels, calculation of PK parameters and, when appropriate, dose adjustment recommendations) was performed in 88.9% (16/18) of our cohort. After the age/weight-based first dose recommendation, only 28% of patients were within the Bu Css target range (on day 1). However, after Bu TDM and dose adjustment recommendations, 75% of patients were successfully within the Bu Css target range. A 4.2-fold variability of Bu Css was observed after receiving the first Bu dose (Fig. 1A: mean Css of 803 ± 347 ng/mL). Css narrowed to exhibit only 2.3-fold variability after TDM dose adjustment (Fig. 1B). The mean AUC$_{cum}$ (16 doses) was 17,224 ± 3040 µmol·min/L, ranging between 13,112 and 22,977 µmol·min/L and representing 1.7-fold intervariability. No AUCs were higher than the targeted AUC$_{cum}$ range, whereas 4 out of 18 patients were under the lower limit, with a mean AUC$_{cum}$ of 13,579.1 ± 490 µmol·min/L (Fig. 1C) and a mean age of 6.3 ± 4.4 years.

The majority (72.2%) of patients required at least one Bu dose modification. A lower dose was recommended in 50% of cases (mean dose decrease −26% ± 12%). To identify patients requiring dose adjustment, the dose ratio between the ninth dose (day 3) and the initial ones was plotted by age (Fig. 1D). Interestingly, dose reduction was observed for children aged 5 years and above. In this subgroup, the mean Css was 1068.5 ± 373.2 ng/mL on day 1. After a single dose modification on day 2 (n = 2) or day 3 (n = 5), no further adjustments were necessary, and all those patients were within the target AUC$_{cum}$ (mean AUC: 19,410.8 ± 2568.3 µmol·min/L). The risk of aGvHD was significantly associated with the last measured Bu Css, with higher Css levels (1011 ± 272 ng/mL, n = 3) in the group developing aGvHD than in the group that did not (771 ± 141 ng/mL, n = 15; *P* = 0.031).

Bu Quantification Method and Process Reliability

QC samples were included in all our routine TDM analyses. The 5-year follow-up chart is shown in Figure 2A illustrating the robustness of the long-term method's performance for the analysis of clinical samples. The mean biases

TABLE 1. Patients' Characteristics (n = 18)

Characteristics	Patients (n = 18)
Age at transplantation (yrs)	7.1 ± 4.1
Weight (kg)	27.3 ± 15.1
Diagnosis	
Malignancies (%)	
Acute myeloid leukemia	27.8
Acute lymphoblastic leukemia	16.7
Myelodysplastic syndrome	22.2
Myeloproliferative syndrome	5.6
Nonmalignancies (%)	
Hemophagocytic lymphohistiocytosis	11.1
Hemoglobinopathy	11.1
Anemia	5.6
HLA compatibility (%)	
HLA-identical sibling	33.3
Matched unrelated donor	22.2
Mismatched unrelated donor	44.4
Stem cell source (%)	
Bone marrow	27.8
Cord blood	50.0
Peripheral blood stem cell	22.2
Myeloablative conditioning* (%)	
Bu and CY	61.1
Bu, CY, and Mel	5.6
Bu and Flu	5.6
Flu and Bu	11.1
Flu, Bu, and Mel	11.1
Flu, Bu, and Thio	5.6
Initial dose/weight (mg/kg)	0.8 ± 0.1
Bu dose 1 (mg)	21.6 ± 11.3
Bu dose 5 (mg)	23.4 ± 13.6
Bu dose 9 (mg)	19.5 ± 11.4
First Bu dose modification (%)	
At dose 3	66.7
At dose 4	20.0
Between doses 5 and 7	13.3
Bu dose change† (last dose/dose 1)	0.9 ± 0.4
Bu dose variation of more than 10% of the original dose‡	
Increase (%)	22.2
No change (%)	27.8
Decrease (%)	50.0
Bu levels after initial weight-based Bu dose	
Css (ng/mL)	803 ± 346.7
AUC‡ (mg·h/L)	4.8 ± 2.1
Bu levels after the last TDM§ and Bu dose recommendation	
Css (ng/mL)	812.0 ± 184.3
AUC‡ (mg·h/L)	5.0 ± 1.2
AUC_{cum}¶ (µmol·min/L or mg·h/L)	17,224.0 ± 3040.6 or 70.7 ± 12.5
Overall survival‖ (%)	50
aGvHD (%)	17
SOS (%)	17

TABLE 1. (*Continued*) Patients' Characteristics (n = 18)

Characteristics	Patients (n = 18)
Mortality (%)	11
Relapse or progression (%)	22
Neutrophil recovery (d)	20.7 ± 10.8

Data expressed as mean ± SD or as percentages.

*Bu, busulfan; CY, cyclophosphamide; Flu, fludarabine; HLA, human leukocyte antigen; Mel, melphalan; Thio, thiotepa.

†This presents the last Bu dose change after TDM and the subsequent dose recommendation; the last dose change was performed at dose 5 (n = 10) or dose 9 (n = 5).

‡This presents the AUC extrapolated to infinity from dosing time, based on the last observed concentration (ie, AUC_∞_obs).

§The last measured TDM was performed at day 3 (n = 12), day 2 (n = 4). Only 2 patients had a single TDM on day 1.

¶The cumulative Bu exposure is the sum of the dose of each Bu amount (mg) multiplied by its related measured AUC from dosing to last concentration ($AUC_{0-\infty}$).

‖aGvHD ≥2 (McDonald criteria), death, hepatic SOS (Seattle criteria and ultra-sound confirmation), relapse or progression. See the method section for details on the diagnostic criteria.

were 0% ± 10%, −2% ± 14%, and 0% ± 12% for Bu QC samples at 300, 600, and 1400 ng/mL, respectively. The QC levels generally fell within the ±15% variability around the target value, although 6%, 9%, and 9% did not fall within these limits for the 300, 600, and 1400 ng/mL samples, respectively. When considering the 3 QC levels together (n = 105), 8% were outside the FDA's ±15% acceptance limit and 6% were beyond ±2 SD. The outlier QC samples were randomly distributed and at least 2 QC samples per series were within ±15%.

Cross-Validation of Bu Analytical Methods

Twenty-one TDM centers around the world independently reported their results for the quantification of 8 blinded QC samples, and these were compared with the theoretical values (Fig. 2B). Laboratories E, Q, R, and J (19%) lacked Bu specificity. Five laboratories had to test a second QC batch (shown as F*, J*, O*, P*, and Q* in Fig. 2B) and 2 (J and Q) were defined as not cross-validated. Bu exposure–prediction based on unreliable methods is of concern because it could lead to inadequate Bu dose adjustments (see **Table S1, Supplemental Digital Content 1**, http://links.lww.com/TDM/A231).

Long-Term Bu Stability

Bu plasma stability showed a mean loss of 14.9% ± 13.4% from the initial Bu concentrations when stored for up to 4 years. The observed biases by storage duration were −20% ± 5% (n = 5, 5 years), −15% ± 8% (n = 22, 4 years), −15% ± 10% (n = 30, 3 years), and −14% ± 9% (n = 19, 2 years). According to the duration of storage, the correlation coefficient r^2 varied from 0.86 to 0.96 (see **Figure S1A, Supplemental Digital Content 1**, http://links.lww.com/TDM/A231), and the overall correlation between repeat and original analyses >2 years was r^2 = 0.92 (n = 76). The mean biases for storage from 12 to 24 months and for up to 12 months were found to be −3% ± 2% (n = 10, r^2 = 0.98) and −1% ± 1% (n = 5, r^2 = 0.99), respectively.

Bland–Altman plots were constructed with all samples in storage for more than 2 years (see **Figure S1B,**

Supplemental Digital Content 1, http://links.lww.com/TDM/A231). Only 5.6% of these samples were outside the limits of agreement. A negative bias of 90.5 ng/mL (corresponding to 11.7%, see **Figure S2**, **Supplemental Digital Content 1**, http://links.lww.com/TDM/A231) was observed over the period studied (2011–2013), which indicates the satisfactory stability of Bu in plasma stored at −80°C. Only a few specimens (n = 20) were stored as DPS on Whatman paper during the period studied. This storage technique led to specimen instability (mean bias −35% ± 19%, n = 20 and up to −58% beyond a 4-year period). Correlation between the original and repeat measures was $r^2 = 0.73$ (see **Figure S1C**, **Supplemental Digital Content 1**, http://links.lww.com/TDM/A231). The Bland–Altman plot showed that the data points were within the limits of agreement (see **Figure S1D**, **Supplemental Digital Content 1**, http://links.lww.com/TDM/A231).

## DISCUSSION

Pediatric Cohort

TDM-based dose adjustment improved Bu exposure in the pediatric patients at our center. This suggests that the implementation of rapid analytical methods for Bu TDM could help to achieve an earlier first dose adjustment, verification, and prompt readjustment within the first few doses. The cumulative AUCs for these children were below the upper limit of the targeted range, and more than 70% of these children required at least one Bu dose modification, mostly dose reductions (>50% of children). Children aged above 5 years mostly required dose reduction, supporting the hypothesis of a decreased elimination of Bu in these children, because of the reduced function of glutathione S-transferase alpha 1 (GSTA1).[38] We confirmed an important variability in Bu exposure in children after initial weight/age-based dose recommendations.[10] Only 28% of our pediatric cohort were within the plasma target range using conventional age/weight approach that is in line with the previously reported 24.3% (studied in 729 children)[39] to 26% (studied in 34 children).[14] This highlights the concern of achieving target AUC or Css in children. Hence, several guidelines were developed, but the performance of these guidelines to predict accurate IV Bu first dose to attain target AUCs was reported with notable differences among them.[10,40] On average, approximately 49.5% of the children (n = 101) were predicted to achieve target AUCs using existing guidelines including Bayesian models to estimate IV Bu first dose.[40] Moreover, performance testing of these guidelines showed that only 13%–16% of the children may achieve target AUCs on calculating dose by a dosing guideline based on actual bodyweight or body surface area,[40] which is lower than the observed rate in our cohort. Although Bayesian dosing models[18,19,30] could achieve target AUCs in about 50%–70% of the patients, implementation of these models in routine is challenging for various reasons, for example, such as unavailability of user-friendly tools. Moreover, it is also essential to verify the dose adjustment by TDM. The necessity of obtaining target AUC and verification after adjustment is even greater when once-daily dosing is

adopted for Bu. In this study, we highlighted the clinical importance of Bu TDM in pediatric population. Through this approach, 75% of children experienced an improvement in their Bu exposure and benefited from a dose adjustment guided by TDM. Likewise, in other cohorts, TDM has been shown to be necessary to achieve Bu target ranges in 40%–68% of patients.[5,17,34,41,42]

The large interindividual variability in Bu exposure in pediatric patients may depend on several factors, such as age, sex, disease, co-medication, and genetic variations. Several studies have shown that polymorphism in a gene encoding the main Bu metabolism enzyme, GSTA1, is associated with Bu PK and clinical outcomes.[34,43,44] The variability in Bu exposure has also been reported to be mainly due to age-related Bu clearance.[45] Indeed, the maturation and activity of the GSTA1 enzyme during the 2 first years of life[4] or up to a bodyweight of 9 kg,[46] has been linked to an alteration in Bu's bioavailability. Population PK models have also shown promising results in the personalization of pediatric Bu dosing. One simulation based on a nonlinear weight-based dosing strategy predicted a 71%–78% probability of achieving the target AUC range.[18] The addition of *GSTA1* genetic information into these population Pediatric population PK models showed improvement for an accurate initial dose prediction giving a level within target AUC range.[40] However, PK models based on small cohort might be difficult to transpose to other cohorts and require advanced mathematical modeling skills. Thus, most centers apply the simple weight-dose recommendations. Bu clearance has been reported to decrease on the second day of Bu administration.[18] This could be related to the temporary dose increment in 2 patients at the fifth dose (day 2, mean fifth dose of 23.4 ± 13.6 mg compared with the initial dose of 21.6 ± 11.3 mg, Table 1 and see **Figure S3**, **Supplemental Digital Content 1**, http://links.lww.com/TDM/A231). The saturation of metabolic capacities in carriers of some genetic variants of *GSTA1*,[38] together with the depletion of glutathione could be other reasons. After a further dose adjustment, a lower interpatient variability and higher percentage of patients within the target range were observed at the last measured Css, especially in young children (see **Figure S4**, **Supplemental Digital Content 1**, http://links.lww.com/TDM/A231).

The benefits of TDM on more than one occasion to keep Bu levels within the target range are encouraging, notably regarding treatment-related toxicity and complications in the Bu regimen. The Bu-based conditioning regimen before HSCT is an important step toward reducing the burden of the underlying disease and easing the engraftment. However, the management of adverse events related to overexposure, such as SOS and aGvHD, or to underexposure, such as relapse and graft failure, can be challenging. This study confirmed the link between high Css and aGvHD reported by others.[5,12,14] Few studies have recorded the dose variations throughout Bu conditioning; however, increased doses after TDM have often been reported because initial doses were insufficient to reach the recommended therapeutic window.[14,17,39] On the contrary, we observed that 9 out of 18 children had their doses reduced after TDM, resulting in 33%,

FIGURE 1. TDM of Bu in a pediatric cohort (n = 18) at Geneva University Hospitals. IV Bu was administered 4 times per day for 4 days, as a 2-hour infusion. A, all steady-state Bu plasma concentrations (Css, ng/mL) were measured by Geneva University Hospitals' Clinical Pharmacology Unit. Up to 3 TDM measurements were taken per patient, followed by a subsequent Bu dose recommendation (n = 41). The blue rectangle represents the Bu Css conventional therapeutic window: 600–900 ng/mL. B, Bu Css after a weight-based dose (day 1) and the postdose adjustment after TDM. Pediatric patients' follow-up for the Bu conditioning regimen at a frequency of 4 doses per day. C, the total Bu exposure after the 16 Bu doses is expressed as the cumulative Bu AUC, from the time of the first dose to the last measurable concentration (μmol·min/L). The blue rectangle indicates the conventional therapeutic window for the cumulative AUC for an adult: 14,400 to 24,000 μmol·min/L (corresponding to a cumulative AUC of 59.12–98.52 mg·h/L) and 75% of the patients in our cohort achieve the targeted cumulative Bu AUC. D, the fold change of Bu dose represents deviations from the intercept (reference: weight-based dose 1 at day 1) at dose 9 (day 3). The gray zone shows the ±10% dose variation of the original dose. The dose changes are shown by age. Please refer to the online version of the article for color figures.
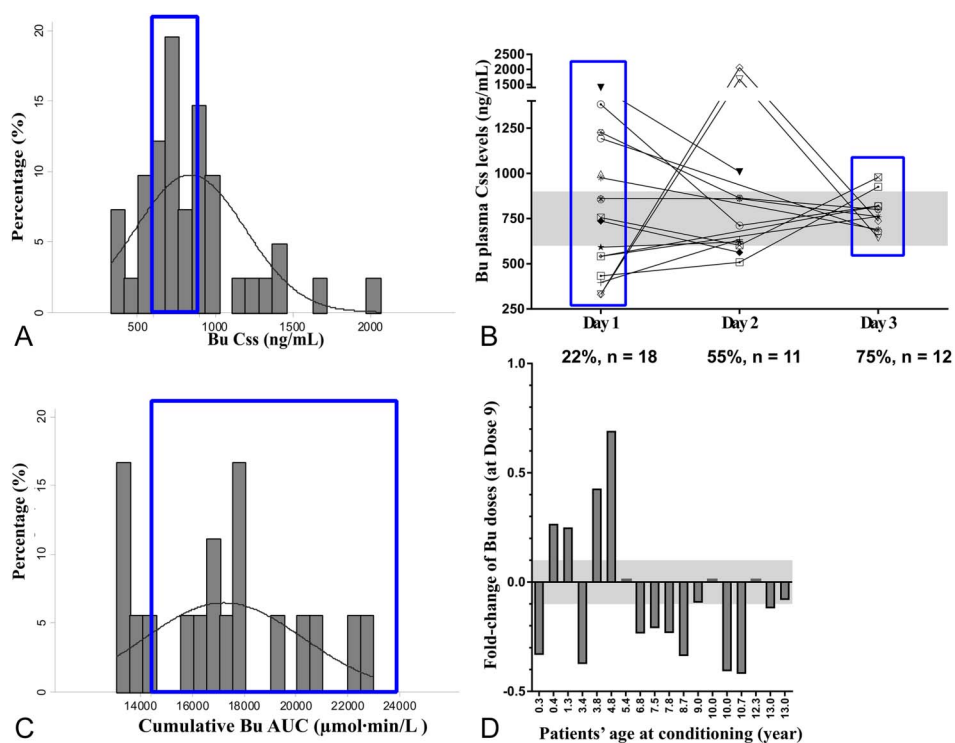
27%, and 0% of patients having suboptimal Bu Css levels on days 1, 2, and 3, respectively.

Several studies have attempted to define the optimal Bu exposure in children. One of the largest recent studies, involving 674 pediatric patients, defined an optimal target AUC_{cum} of 18,963–24,555 μmol·min/L, irrespective of the indication. We observed a similar range, with an AUC_{cum} of 17,188.5 ± 3038.9 μmol·min/L. Higher levels, AUC_{cum} >24,555 μmol·min/L (equivalent to a Css of 1052.1 ng/mL), have been associated with treatment-related toxicity.[12] Considering that 50% of our pediatric cohort required a reduction in their Bu dose after the TDM recommendation, one cannot exclude that using age/weight-based dose recommendations only may raise the risks of high Bu exposure and overdose. The observations presented in this study were based on a limited number of participants, and factors other than Css or AUC_{cum} should be investigated. For instance, well-known risk factors such as diagnosis, the sequence of administration (Bu/Cy, Cy/Bu), preexisting liver damage, drug–drug interactions, and the intrinsic amount of antioxidant glutathione may contribute to Bu toxicity.[47–49]

Performance of the Bu Quantification Method, Cross-Validation of Bu Analytical Methods, and Bu Stability

The reliability and performance of the method of Bu analysis used at our center for 5 years was verified by the

internal QCs included in each routine analysis and by the external QCs. Isolated deviations were detected (change of calibrator batches, operators, or internal calibration of the instrument) at the time of analyses and samples were rerun if needed. The long-term performance of the method was assessed at several levels included in routine analyses. In addition, the overall accuracy was ensured by the external QC program. In that context, a cross-validation of the Bu methods used by the 21 participating TDM centers around the world demonstrated that 19 out of 21 methods were efficient and accurate. A total of 5 centers produced unsatisfactory result after the first cross-validation exercise, and these centers used several quantification methods (from UV to MS/MS methods). A second set was then sent to rule out any shipment issue or any temporary method deviation (unforeseen error limited in time). The 2 laboratories that failed the cross-validation in the second round used both UV detection methods. They both overestimated Bu (mean biases of 54.3% and 26.6% in the second round for laboratories J and Q, respectively). So far, these 2 centers have developed a new analytical method and are now in the process of validation.

High-performance liquid chromatography coupled with UV detection was previously considered the gold standard analytical method for detecting Bu. However, it is reported to have limited selectivity. The UV detection method could suffer deleterious matrix effects as isotopically labeled Bu cannot be used as the internal standard. This implies an extraction process that is more time-consuming. In addition, UV detection methods for Bu quantification often include an additional step of

FIGURE 2. Long-term (>2 years) Bu stability in plasma and DPS, QC, and accuracy of the Bu quantification process. A, the QC chart of the Bu quantification process routinely performed for the TDM of pediatric patients. Five-year follow-up (35 series × 3 levels) of Bu QC plasma samples spiked at 300, 600, and 1400 ng/mL. The horizontal solid lines indicate the mean values, the horizontal dashed lines indicate the limit of ±2 SD, and the gray zone indicates the US FDA acceptance limits of ±15%. QCs and calibrators were freshly prepared for each measurement. Across the whole series, 4, 8, and 7 samples were outside the limits for the 300, 600, and 1400 ng/mL QC levels, respectively, and these were distributed randomly along the series. In such cases, additional analyses and assessments of the accuracy were performed. B, cross-validation of Bu quantification processes: General results from the Bu TDM centers around the world participating in the ALL SCTped Forum study in fall 2015. The 21 sets of assay results from the QC samples of Bu levels (blinded to centers) were independently reported to our laboratory and compared against the theoretical values for each sample. Five laboratories (23.8%) had to test a second set of QCs, shown as "*." Results were within the criteria for acceptance except for 2 centers, shown as Lab J, J* and Q, Q*, representing their first and second QC sets, respectively. The horizontal dashed lines represent the limit of ±15% difference from the given results and the labeled concentrations.
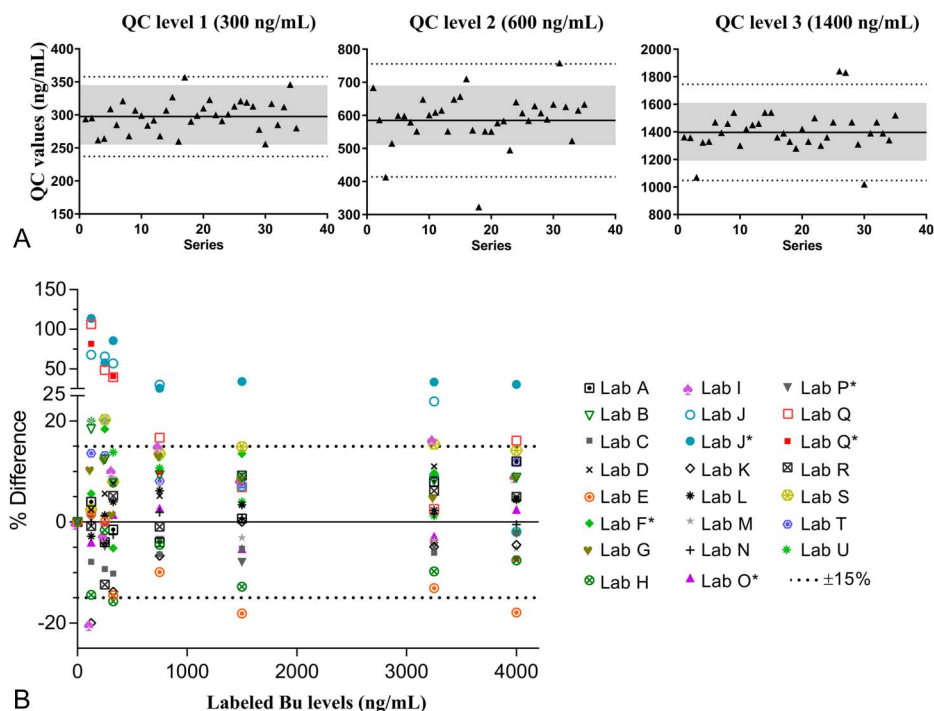
derivatization, which could add a source of variability and risks of human errors. Furthermore, imprecision in the preparation of calibrators, long-term storage of calibrators, and lack of Bu stability after some freeze–thaw cycles may affect the results as well. Nowadays, despite its high cost, LC–MS/MS is considered as the gold standard analytical procedure for TDM because of its robustness, specificity, and high sensitivity. The LC–MS/MS methods allow for a fast throughput and avoid other sources of variability (i.e., derivatization procedure for UV method for drugs such as Bu and time-consuming sample extraction procedures). In addition, to assess the accuracy of an analytical method, the cross-calibration exercise gives the opportunity to pinpoint the inadequate procedure and to improve practices of the centers that failed the test. Such validation exercises shall be often conducted to maintain quality assurance. Thus, this exercise conducted for the first time on a large-scale and with a large number of Bu TDM centers can help to improve and ensure the quality of the results across all centers.

In this study, we simulated hypothetical Bu dose calculations based on the Bu levels estimated by unreliable analytical methods. PK parameters (AUC and terminal elimination rate constant lambda-z) and subsequent dose adjustments were calculated by simulating the Bu exposure of 1 adult and 2 pediatric patients using the mean instrumental bias observed for the 2 underperforming laboratories. Simulated Bu doses from these 2 laboratories were of concern and found to be 50% lower than the actual doses required to attain the target window (see **Table S1B**, **Supplemental Digital Content 1**, http://links.

lww.com/TDM/A231). This simulation did not take into account the expertise of hospital teams, however, and this factor may counteract the risk of this kind of technical issue. The quantification methods used by each of the participating centers are listed in **Supplemental Digital Content 1** (see **Table S2**, http://links.lww.com/TDM/A231). This cross-validation exercise highlighted that the Bu levels measured were not significantly different in 19 out of 21 participating ALL SCTped Forum study centers across the world.

With reference to stability, Bu was found stable in plasma stored at −80°C for 4 years. These data add to existing knowledge on Bu stability[28,29] and are especially useful in retrospective clinical studies with low recruitment rate. This study only tested one type of DPS card; further studies are required to assess other DPS types. Because degradation of a DPS dry matrix is limited,[50,51] the lower long-term stability of Bu on a DPS than in plasma from the same source could not be explained. However, from our observations, it is not recommended to store Bu in the studied DPS card.

CONCLUSIONS

The present report illustrated the clinical importance of performing TDM of the IV Bu treatments given to pediatric patients. Our observations demonstrated some clinical advantages of personalizing Bu doses for children using TDM throughout the dosing schedule (dose adjustment and verification of adjusted doses) to better achieve the target range for the

drug in plasma. Most of the participating TDM centers accurately quantified Bu, as illustrated by cross-validation testing. Both the centers that failed the cross-validation had been using UV detection. The study's observations also highlighted the importance of internal and external QC sample analyses to verify the long-term performance of the quantification method used and the consistency of results in a particular TDM center. Finally, we showed that Bu stability in plasma stored at −80°C was adequate for up to 4 years.

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