SHORT COMMUNICATION

Comparison of Two Analytical Methods for Busulfan Therapeutic Drug Monitoring

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Abstract

Background and Objectives Busulfan (Bu) is an old drug, but is still well recommended as an alkylating agent during conditioning therapy, before hematopoietic stem cell transplantation. Although its dose administration is standardized and based on patient weight, therapeutic drug monitoring is required in order to maintain its exposure [as area under the concentration-time curve (AUC) from 0 to infinity $AUC_{0-\infty}$] within a narrow therapeutic range and, if necessary, to adjust the dose with as short a lead time as possible. The aim of the study is to evaluate the agreement (as calculated AUC) between a gold standard analytical method and a new one that is faster and easier.

Methods We analyzed 221 plasma samples from 37 children (0.25–16 years; 4–62.5 kg) and 11 adults (21–59 years; 45–80 kg), corresponding to 52 AUC values (ng h/mL). The drug exposure was calculated, simultaneously, by two validated analytical methods. The reference method was a high-performance liquid chromatography (HPLC) assay combined with an ultraviolet detector (UV). The test method had a triple quadrupole mass spectrometer (MS) as detector; the clean-up procedures of the samples were different and faster.

Results The agreement between the two methods (reference and test) was evaluated in terms of Bu exposure differences based on Lin's concordance correlation coefficient (CCC) and represented by the Bland–Altman plot. The CCC between the AUC of the two methods was excellent (0.868; 95% CI: 0.802–0.935). The precision of the measures (expressed by Pearson's *italic "r"*) was 0.872, and the accuracy (accounted by the bias correction factor) was 0.996.

Conclusions We can conclude that the HPLC–MS/MS assay represents a very good alternative to the reference.

Key Points

Busulfan therapeutic drug monitoring is still strongly recommended before hematopoietic stem cell transplantation.

The widely accepted analytical procedures (HPLC–UV) to detect busulfan plasma exposure require time-consuming steps and a large volume of blood, especially for the pediatric population.

A faster and easier HPLC-MS/MS analytical method is proposed; the agreement between the two methods is excellent.

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1 Introduction

Busulfan (Bu) is a DNA alkylating agent that is widely used in conditioning therapy for hematopoietic stem cell transplantation (HSCT). Oral Bu was widely replaced by an intravenous formulation (IV Bu) [1–3] based on body weight alone, regardless of age. This route of administration has significant advantages in terms of high rate of sustained engraftment, low transplant-related mortality and promising survival outcomes post-transplant. IV Bu is well tolerated in the particular context of HSCT and its failure due to organ toxicity was seldom reported. Sometimes failure is associated with elevated liver enzymes but, in contrast with the oral formulation, is neither related to severe hepatic veno-occlusive disease nor to death as a result of organ toxicity. However, wide inter-individual variability in pharmacokinetics, pharmacodynamics and systemic exposure is still observed, mostly in children [4–7], which is perhaps due to their individual hepatic metabolism, age, diagnosis, pretreatments and concomitant treatments [8–11]. Assessing drug exposure of young

patients still represents a major issue for improving the efficacy and safety of the Bu-based conditioning regimen. If the Bu plasma area under the curve ($AUC_{0-\infty}$) levels are too low, graft rejection or relapse may occur; conversely, if the $AUC_{0-\infty}$ levels are too high, this can lead to an increase in the risk of toxicity, mucositis, hepatic sinusoidal obstruction syndrome and acute graft versus host disease, which could also be fatal [12, 13].

Bu therapeutic drug monitoring (TDM) is therefore mandatory; however, because it requires the collection of multiple blood samples, analyses of small volumes are highly recommended, mostly for pediatric patients.

In order to make Bu TDM less invasive, faster and easier, we developed and validated a high-performance liquid chromatography (HPLC) MS/MS analytical method to replace or support our old, accurate, robust reference assay, that successfully passed all the phases of an international multicenter Bu cross-validation [14], and is regarded as our gold standard analytical method. To investigate the agreement in estimated AUC values, Lin's concordance correlation coefficient (CCC) was calculated and a Bland–Altman plot was created.

2 Materials and Methods

2.1 Patient Cohort

Pharmacokinetic data from 37 children (median age 5 years) and 11 adults (median age 47 years), scheduled for HSCT and in the Bu-based conditioning regimen, were considered for this study. The doses of IV Bu (mg/kg) varied according to the weight of the pediatric patients: < 9 kg → 1 mg/kg; 9–16 kg → 1.2 mg/kg; 16–23 kg → 1.1 mg/kg; 23–34 kg → 0.95 mg/kg, and > 34 kg → 0.8 mg/kg. IV Bu was infused via a central venous catheter over 2 h. Blood samples were collected from a peripheral vein immediately before and at 2, 3, 4 and 6 h after the first dose of Bu. Five patients received a Bu dose < 0.8mg/kg.

Forty patients were administered with a 2-h IV Bu infusion 4 times a day, for a total of 16 doses; the other patients (8) received Bu orally (1 mg/kg/dose: 4 doses/day). Bu doses have to be adjusted in order to reach the target plasma concentration range of 600–900 ng/mL at the steady state (C_{ss}), equivalent to 3,600–5,400 ng h/mL ($AUC_{0-\infty}$). Bu exposure was also calculated as AUC on day 1 (after the first dose) and on day 2 (after the fifth dose) for 4 patients who needed a dose adjustment.

Patients or their relatives provided written informed consent for the processing of personal data, as well as for research purposes, because the Fondazione IRCCS Policlinico San Matteo is a health and research institute. The Bioethics Committee of Fondazione verified the correct data collection in the informed consent.

2.2 Busulfan Quantification in Plasma Samples

Bu dose individualization was performed by taking 5 whole blood samples at fixed time points (at t_0, t_2, t_3, t_4 and t_6 from patients receiving IV infusion at t_0, t_1, t_2, t_4 and t_6 after per os administration). The blood samples were then centrifuged and the separated plasma specimens were processed and analyzed twice, simultaneously; 300 μL were examined by an HPLC–UV system, and 100 μL were analyzed by an HPLC–MS/MS analytical assay.

Bu determinations by the HPLC–UV system were obtained from 300-μL plasma samples spiked with 25 μL of methanol containing 1,6-bis(methanesulfonyloxy)hexane as internal standard (22 μg/mL), subjected to protein precipitation, then to derivatization with sodium diethyldithiocarbamate (Fig. 1), and finally extracted with ethyl acetate. The extracts were dried under nitrogen and reconstituted with 150 μL of mobile phase prior to HPLC determination. Chromatographic separations took place within a Zorbax SB C18 (4.6 × 75 mm 3.5 μm) analytical column, combined with guard columns made of identical packing materials (Zorbax SB C18); the mobile phase was a solution of methanol–water (80:20, v/v) with a flow rate of 1.0 ml/min and a run time of 7 min. The wavelength of detection was set at 251 nm. The lower limit of quantification (LLOQ) was 66 ng/mL. Calibration curves were linear from 66 to 5,280 ng/mL.

At the same time, Bu concentrations were also quantified from 100 μL of plasma samples that were deproteinized with 200 μL of methanol containing busulfan-d8 (Bu-d8) as internal standard (100 μg/mL). Separation and detection of Bu were achieved with a Kinetex® 2.6 μm C18 column (100 × 4.6 mm) maintained at 40 °C, and coupled with a TSQ Quantum Access (Thermo Scientific) triple-quadrupole mass spectrometer as detector. Mobile phases A (ammonium acetate 2 mM in water) and B (ammonium acetate 2 mM in methanol) were both acidified with formic acid (0.1%) and freshly prepared; elution was carried out in gradient mode. The gradient was started at 40% B, linearly increased to 80% B during 0.5 min, maintained for 2.8 min, and reduced to 40% B during 0.05 min. The mobile phase was then returned to 40% B for 1.15 min for re-equilibration. The flow rate was 0.7 mL/min and the total run time was 4 min. The injection volume was 10.0 μL and LLOQ was 31 ng/mL. Bu and Bu-d8 were detected as ammonium adducts in multiple reaction monitoring mode at m/z 263.9 →151.94, 246.87 and 271.9 → 159.94, 254.87, respectively. The calibration concentration ranges (31–2,000 ng/mL) were selected to cover the Bu concentrations expected for therapeutic maintenance.

Xcalibur 2.07 and LCquan 2.5.6 software from Thermo Fisher Scientific were used for the HPLC–MS/MS system
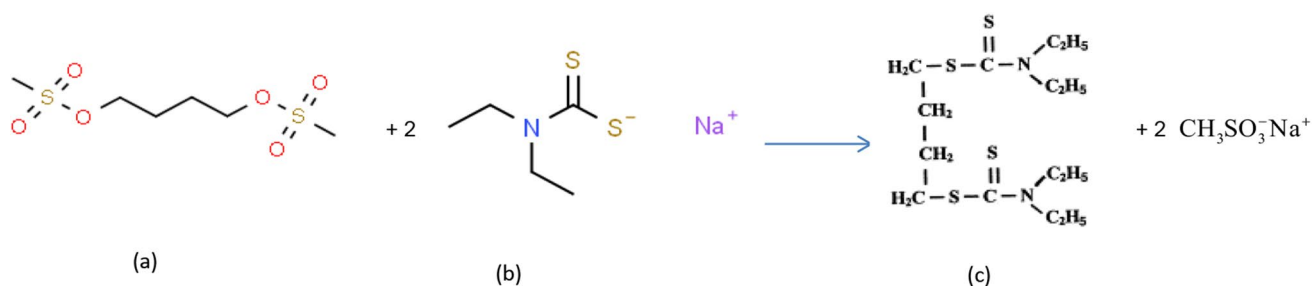
Fig. 1 Stoichiometric reaction between busulfan (**a**) and sodium diethyldithiocarbamate (**b**) to obtain 1,4-bis (diethyldithiocarbamoyl)butane (**c**)

control, data acquisition, and data analysis. The calibration curves were generated from weighted ($1/x$) linear regression curves. Analyte peaks were identified through a combination of retention times and the specific multiple reaction monitoring transitions. The corresponding amounts were quantified by normalizing the peak area to the IS (area analyte/area IS), and concentrations were calculated from the respective calibration curves.

Both the analytical methods were developed and validated in accordance with the European Medicines Agency (EMA) "Guidelines on bioanalytical method validation" [15].

2.3 Pharmacokinetic Analysis

To avoid toxicity while ensuring Bu dose adequacy to completely ablate the bone marrow, IV dosing was guided by a pharmacokinetic evaluation of the AUC after the first dose. The pharmacokinetic evaluation was carried out at the end of the first dose, with results of pharmacokinetic testing available to facilitate dose adjustment before beginning the fifth dose.

Bu exposure (as AUC) was analyzed twice (HPLC–UV; HPLC–MS/MS) for each patient, according to a non-compartmental model and the pharmacokinetic parameters were analyzed by WinNonLin (Pharsight-Phoenix for WinNonLin, Version 6.2.1) validated software.

2.4 Statistical Analysis

The concordance between the two analytical methods (HPLC–MS/MS and HPLC–UV) was performed by means of Lin's CCC that measured how strong the relationship was between the corresponding calculated AUC values [16]. The CCC combines measures of both precision and accuracy to determine how far the observed data deviate from the line of perfect concordance (i.e., the line at 45° on a square scatterplot).

Lin's CCC increases in value according to the nearness of the data's reduced major axis to the line of perfect concordance (the accuracy of the data) and to the tightness of the data about its reduced major axis (the precision of the data).

The CCC ranges in values from 0 to + 1. A CCC value of 0 indicates that most of the error originates from differences in measurements. As CCC values approach 1, the measurement differences between the different methods are becoming negligible and more consistent. Concordance is classified as poor (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), good (0.61–0.80), or excellent (0.81–1.00) [16]. This correlation studies the relationship between the corresponding AUC calculated by the two different analytical methods, but not the differences; the Bland–Altman plot analysis is a simple way to evaluate a bias between the mean differences, and to estimate an agreement interval, within which 95% of the differences of the second method fall, compared to the first one [17, 18]. These statistical limits are calculated by using the mean and the standard deviation(s) of the differences between two measurements. The related graphical analysis (scatter plot) shows the ratio (expressed as a percentage) of the difference between two corresponding AUC values to their means plotted against their means. Horizontal lines are drawn at the mean difference, and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences; 95% of the data points should lie within $\pm$ 2s of the mean difference. $P < 0.05$ was considered statistically significant; all tests were two-sided.

Data analysis was performed with the STATA statistical package (release 13.1, 2014, Stata Corporation, College Station, TX, USA).

3 Results

We compared 52 AUC values, calculated on data from two analytical methods––a gold standard (HPLC–UV) and a new one (HPLC–MS/MS). Both methods were previously validated in accordance with the EMA guidelines [15] and all required criteria were met. The HPLC–MS/MS analytical method has two main advantages—it is faster than HPLC–UV, since the time-consuming derivatization steps during samples preparation are no longer necessary and, mostly, it requires a smaller volume of plasma samples
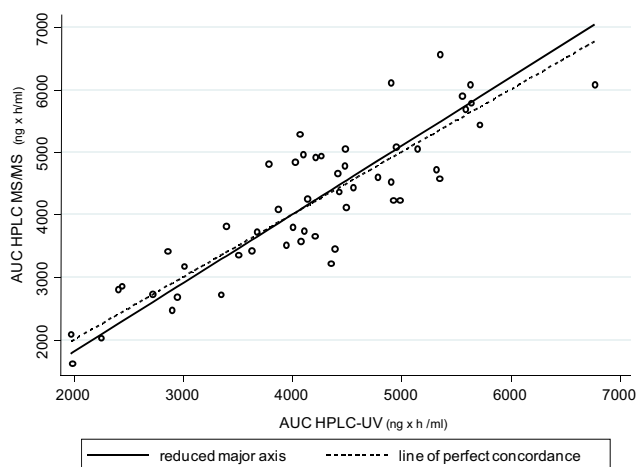
Fig. 2 Linear regression plot of AUC HPLC–MS/MS vs AUC HPLC–UV. *AUC* area under the curve, *HPLC–UV* high-performance liquid chromatography–ultraviolet light, *HPLC–MS/MS* high-performance liquid chromatography–tandem mass spectrometry
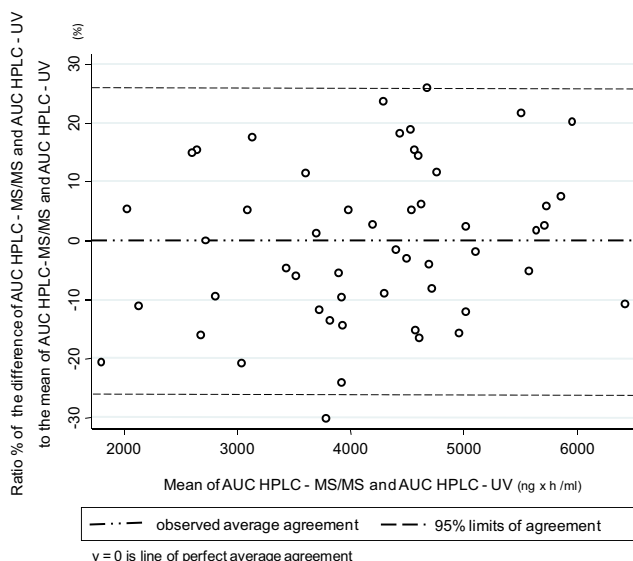
Fig. 3 Bland–Altman plot: ratio, expressed as percentage, of the difference of AUC HPLC–MS/MS and AUC HPLC–UV to the mean of AUC HPLC–MS/MS and AUC HPLC–UV vs their means. *AUC* area under the curve, *HPLC–UV* high-performance liquid chromatography–ultraviolet light, *HPLC–MS/MS* high-performance liquid chromatography–tandem mass spectrometry

(100 µL), which is a significant benefit for the pediatric population.

The estimates of the total systemic exposure to busulfan (AUCs) and the pharmacokinetic parameters were derived by modeling the raw data to fit a non-compartment model.

The agreement between results (calculated AUC values) was evaluated with Lin's CCC and represented by the Bland–Altman plot.

The CCC between the corresponding calculated AUC of the two analytical methods was excellent (0.868; 95% CI 0.802–0.935); the precision of the measures, expressed by Pearson's *r* was 0.872, and the accuracy (accounted by the bias correction factor) was 0.996.

The line of the best fit to the data, comparing the two methods, is shown in Fig. 2. The mean difference between the two methods plotted against the pair-wise mean is shown in the Bland–Altman plot (Fig. 3), indicating that the two methods yield very similar results for pharmacokinetic evaluations.

4 Discussion and Conclusion

Bu is an alkylating agent used to ablate bone marrow cells prior to HSCT for chronic myelogenous leukemia. Oral Bu was widely replaced by an intravenous formulation (IV-Bu), based on body weight, regardless of age; this route of administration has significant advantages in terms of high rate of sustained engraftment, low transplant-related mortality and promising survival outcomes post-transplant. However, wide inter-individual variability in pharmacokinetics, pharmacodynamics and systemic exposure are still observed mostly in children requiring Bu TDM.

In order to make Bu TDM less invasive, faster and easier, we developed and validated an HPLC–MS/MS analytical method to replace or support our old, accurate and robust HPLC–UV method, that successfully passed all the phases of an international, multicenter Bu cross-validation [14] and was regarded as our gold standard analytical method.

Bu plasma samples were analyzed twice by two different analytical methods—HPLC–UV and HPLC–MS/MS.

The Bland–Altman plot (Fig. 3) indicates that the two methods yield very similar results for pharmacokinetic evaluations.

The two analytical methods are precise, sensitive and accurate; their agreement in terms of calculated AUC is excellent, but HPLC–MS/MS avoids the time-consuming sample derivatization step and requires a smaller sample volume. This analytical method is well recommended for the rapid and accurate measurement of Bu in clinical drug monitoring, especially for pediatric patients, although it is known that not all clinical laboratories could invest in a dedicated mass spectrometer and in co-workers with in-depth technical knowledge.

Conflicts of Interest Authors Simona De Gregori, Antonella Bartoli, Carmine Tinelli and Federica Manzoni declare that there is no conflict of interest.

Ethics Approval and Informed Consent The study did not require any specific ethics approval. The Fondazione IRCCS Policlinico San Matteo is a health and research institute: the patients or their relatives provided written informed consent for the processing of personal data, as well as for research purpose. The Bioethics Committee of Fondazione verified the correct data collection in the informed consent.

Consent for Publication I, Simona De Gregori, on behalf of any co-authors, hereby declare that we all participated in the study and in the development of the manuscript titled "Comparison of two analytical methods for Busulfan therapeutic drug monitoring". We have read the final version and give our consent for the article to be published.

Author Contributions A.B. and S.DG. conceived the presented idea, and planned and carried out the experiments. C.T. and F.M. processed the experimental data, designed the figures and aided in interpreting the results. A.B., S.DG., C.T. and F.M. contributed to the writing of the manuscript. All the authors have accepted responsibility for the entire content of this manuscript and approved submission.

Availability of Data and Material All data and results are stored in the laboratories' database by the Fondazione IRCCS Policlinico San Matteo.

Code Availability Not applicable.

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