AN LC-MS ASSAY WITH ISOCRATIC SEPARATION AND ON-LINE SOLID PHASE EXTRACTION TO IMPROVE THE ROUTINE THERAPEUTIC DRUG MONITORING OF BUSULFAN IN PLASMA

LC-MS ESEJ SA IZOKRATSKOM SEPARACIJOM I ON-LINE EKSTRAKCIJA ČVRSTOM FAZOM U CILJU BOLJEG RUTINSKOG TERAPIJSKOG PRAČENJA LEKA BUSULFAN U PLAZMI

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Summary

Background: Busulfan (Bu) requires therapeutic drug monitoring (TDM) in subjects undergoing a conditioning regimen for hematopoietic stem cell transplantation (HSCT). To speed up the procedure and increase reproducibility, we improved our routine LC-MS/MS assay using the on-line solid-phase extraction (SPE) of samples.

Methods: A protein precipitation (PP) step was performed before the on-line SPE of Bu from 200 µL of plasma spiked with octa-deuterated Bu (D₈-Bu) as the internal standard. Bias was assessed with respect to our routine LC-MS/MS Bu assay with off-line extraction using the Passing-Bablok robust regression. Root cause of bias for individual samples was assessed by analyzing the regression residuals.

Results: The method was linear in the range 37.75–2,416 ng/mL (r²>0.999), with 19.74 ng/mL LLOQ and 10.5% CV at 20 ng/mL. Precision and accuracy were both within ±5%, and neither appreciable matrix nor carryover effects were observed. The Passing-Bablok regression analysis returned a 0.99 slope (95% CI: 0.97 to 1.01) and –6.82 intercept (95% CI: –15.23 to 3.53). Residuals analysis against the 2.5th–97.5th percentiles range showed four samples with significant bias individually.

Conclusions: The method presented can be successfully employed for the routine analysis of Bu in plasmatic samples, and can replace the LC-MS/MS method with off-line extraction without any statistically significant overall bias. In
Introduction

Hematopoietic stem cell transplantation (HSCT) represents the only therapeutic approach to grant blood transfusion-free survival to patients affected by severe thalassemia (1). The high-dose Busulfan (Bu) combined with cyclophosphamide (Cy) regimen is to date successfully employed to eradicate the genetically inherited disorder and permit the engraftment (2). Bu is an alkylating compound formerly developed as an orally administrable drug for myeloproliferative disorders (3). Since then, it has been reformulated as an intravenous (iv) agent aimed at minimizing interpatient as well as dose-to-dose variability (4). However, by virtue of its mechanism of action, Bu exhibits a narrow therapeutic window, and therapeutic drug monitoring (TDM) is thus necessary to overcome toxicity or inefficacy (5).

To date, some antibody-based methods are available for dosing Bu (6, 7). In spite of this fact, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) represents the widespread choice to perform its TDM on several different matrices (8–11). Superiority of LC-MS/MS in immunometric assays relies on its higher specificity, although the extractive steps like liquid-liquid extraction (LLE) or protein precipitation (PP) if required cannot be automated (12). In this regard, solid phase extraction (SPE) represents a valuable technique suitable to achieve an automated extraction of samples within the chromatographic apparatus. Through the column switching technique (CST), it is indeed possible to put an SPE cartridge on-line to an analytical column, to achieve an automated extraction of samples within (13). The on-line SPE through CST allows a reduced time analysis and increased reproducibility, limiting to the minimum the manual intervention in sample cleanup.

In the present study, we report a robust CST-based LC-MS/MS Bu assay with on-line SPE, developed to speed up the TDM of Bu in pediatric patients undergoing a conditioning regimen for HSCT. Alongside, we also introduced the analysis of residuals obtained from the methods agreement regression, in order to recognize individually biased samples and address the potential root cause in relation to the new method.

Materials and Methods

Chemicals and reagents

Busulfan (Bu) was purchased from Sigma-Aldrich (Milan, Italy), while [2H₈]-Busulfan (D₈-Bu, 97.5%) for internal standard (IS) was purchased from Euriso-Top (Paris, France). LC/MS grade acetonitrile (ACN), methanol (MeOH), ethyl acetate (EtAC), and zinc sulfate heptahydrate (ZnSO₄·7H₂O), ammonium acetate (NH₄Ac), sodium hydroxide (NaOH) and formic acid (FoA) were purchased from Sigma-Aldrich (Milan, Italy). Ultrapure water, with a resistivity of 18 MΩ cm, was obtained from a Millipore Milli-Q Ultrapure 8 apparatus (Milan, Italy).

Stock solutions for calibration standards, quality controls and internal standard were prepared in ACN in polyethylene tubes at a final concentration of about 1 mg/mL, calculating the actual concentration on a total volume of 10 mL (for calibration standards and quality control) or 1 mL (for internal standard). Stock solutions for calibration standards and quality controls were obtained by two independent weights of Bu. All stock solutions were stored at –80 °C for no longer than 6 months.

Working solutions for calibration standards (wSTD) were made by a first 1:50 dilution of the stock solution, followed by six serial 1:2 dilutions to obtain a total of 7 calibration points; for quality controls (wQC), a first 1:100 dilution was followed by two 1:2 serial dilutions, while internal standard (wIS) was obtained by a single 1:100 dilution. All dilutions were made with a solution of water/ACN 50:50 (v/v) and stored at +4 °C for no longer than a month. The same working solutions were used for both on-line and off-line methods.

Sample collection

Blood samples were collected in purple-top K₃-EDTA Vacutainer tubes (Becton Dickinson, Milan, Italy), transported refrigerated at +4 °C and promptly centrifuged after arrival at laboratory (1,500 g for 5 minutes). For the same plasma, one sample aliquot was used on the same day to assay Bu by the routine method (with off-line extraction), and thus stored at +4 °C until analysis; conversely, a second aliquot was transferred to a plastic vial and stored at –80 °C to be assayed with the alternative method (on-line SPE extraction). A total of 20 blood specimens from...
healthy drug-free untreated subjects where pooled and used for method development and analytical performance evaluation. Conversely, 80 blood specimens from 5 different pediatric patients were used for validation of the newer Bu assay on real samples. For each patient, four consecutive samples were taken every day at every two hours, over four consecutive days, as previously described (14).

Calibrators, controls and sample preparation
Calibrators and controls were prepared by spiking 200 μL of pooled plasma with 20 μL wIS, and finally adding 20 μL of wSTD or wQC to prepare STDs or QCs respectively. Clinical samples were instead prepared by spiking 200 μL of patient’s plasma with 20 μL of wIS.

Actual concentrations for calibrators used in this setting were in the range of 37.75–2,416 ng/mL (STD7-STD1), and controls were in the range of 310–1,240 ng/mL (QC3-QC1).

On-line SPE LC-MS/MS equipment and conditions
The apparatus for on-line extraction and chromatography comprised a Dionex UltiMate 3000 HPLC system (Sunnyvale, CA, USA) with column oven, a PerkinElmer PE 200 quaternary pump (Waltham, MA, USA) with on-line vacuum degasser and a Valco (Milan, Italy) multiport valve for the column switching.

The PP was performed before on-line SPE by adding 400 μL of 4.5% ZnSO₄ (w/v) + 2 mmol/L NH₄Ac, and then centrifuging at 14,000 g for 5 minutes, with 100 μL of supernatant transferred to an autosampler vial.

The on-line SPE extraction was performed on a Restek Pinnacle II C18 5 μm 10 mm × 2.1 mm SPE cartridge (Bellafonte, PA, USA), while chromatographic separation was achieved on a Restek Pinnacle II C18 5 μm 100 mm × 2.1 mm (Bellafonte, PA, USA), thermostatted at 50 °C. For the SPE extraction, the mobile phase (A) consisted of a solution of 20 mmol/L NH₄Ac/Methanol 95:5 (v/v), while for the SPE elution and isocratic separation on the analytical column, the mobile phase (B) consisted of a solution of 20 mM NH₄Ac + 0.1% FoA/Methanol 20:80 (v/v).

Mobile phases flow, valve positions and timings are reported in Table I. Injection volume was 10 μL.

For detection, an API 4000 Qtrap triple-quadrupole (AB Sciex, Toronto, Canada) equipped with an electrospray ionization (ESI) Turbo V ion source was used in MRM positive mode, choosing the 264.1 → 151.1 m/z and 272.1 → 159.1 m/z mass transitions to monitor the ammonium adducts of Bu and D8-Bu respectively. Nitrogen was used as the curtain and nebulizer gas, and the acquisition parameters were set as detailed in Table II.

Data were acquired and processed with Analyst 1.5.1 (AB Sciex, Toronto, Canada).

Table I Settings for on-line SPE and LC analysis.

<table>
<thead>
<tr>
<th>Run time (min)</th>
<th>Extraction phase (A) flow (mL/min)</th>
<th>Separating phase (B) flow (mL/min)</th>
<th>Switching valve</th>
<th>Valve Position</th>
<th>SPE</th>
<th>Analytical</th>
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</table>

Analytical validation of the on-line SPE Bu assay
Tandem mass spectrometric conditions were optimized using 10 μg/mL Bu and D8-Bu solutions injected at a flow rate of 10 μL/min.

Retention times for Bu and D8-Bu were determined by replicate injections (n = 20) of control plasma samples spiked with Bu or D8-Bu at the final concentration of 500 ng/mL.

The matrix effect was evaluated by accounting changes in the total ion count (TIC) signal intensity of a pure Bu or D8-Bu solution made in MeOH at 0.5 μg/mL, continuously infusing post-column at 10 μL/min through a T-junction while injecting on-line replicate blank samples (n = 10) made of pooled plasma of untreated drug-free subjects (blank matrix, BM) (15).
Linearity was assessed by assaying in quintuplicate seven calibration standards in the actual range of 37.75×2,416 ng/mL, and then checking the goodness of fit of the 1/X-weighted least squares linear regression model in respect to the quadratic and the cubic regression models through the F-test on residuals (16). Limit of detection (LOD) and lower limit of quantitation (LLOQ) were estimated through the analysis of the regression curve, considering the standard deviation of residuals (SDR) and the slope (b), thus placing LOD=3.3 SDR/b and LLOQ=10 SDR/b (17).

To assess the carryover, two independent experiments were carried out according to the Clinical and Laboratory Standard Institute EP10-A3 guideline (18). First, the samples QC3 and QC1 were ran in the sequence QC3-QC1-QC3 (corresponding to the concentrations 310–1,240–310 ng/mL), then the samples BM and QC1 were ran in the sequence BM-QC1-BM (corresponding to the concentrations 0–1/240–0 ng/mL), with both batches assayed in triplicate.

Precision and accuracy were estimated through the intra-run and inter-run variability analysis, by analyzing in quintuplicate for the first day, and in triplicate for the following four days, three levels of Bu (QC1, QC2, QC3) (19). Accuracy was expressed as the relative error (%RE) of the grand mean for each of the Bu levels analyzed, while precision was given as the relative standard deviation (%RSD). Recovery was estimated on the three Bu levels assayed on the first day by comparing their response to that of same samples prepared by spiking Bu and D8-Bu after the PP step (20).

### Off-line Bu extraction LC-MS/MS analysis

The on-line SPE LC-MS/MS Bu assay was compared with the LC-MS/MS Bu assay with off-line PP and LL extraction currently in use at our laboratory for the routine TDM of pediatric patients undergoing a conditioning regimen for HSCT (14, 21). The validation was carried out by analyzing on the same day the plasma specimens collected for clinical testing, as previously described. For the Bu assay by off-line extraction, samples, calibrators and controls were prepared following the same spiking procedure described for the on-line assay. The PP was achieved by the sequential addition of 400 µL of a 5% ZnSO4 (w/v) solution and 600 µL of water, followed by a centrifugation step at 2,500 g for 10 minutes. The supernatant was transferred into a tube containing 100 µL of 100 mmol/L NaOH, and the extraction was done by adding 2 mL of EtAC, thoroughly vortexing and finally centrifuging at 2,500 g for 10 minutes. The upper layer was transferred to a new tube and evaporated under nitrogen stream at 37 °C, then resuspended with 100 µL of 40 mmol/L NH4Ac + 0.1% FoA/MeOH 40:60 (v/v) and transferred to an autosampler vial. The chromatography was performed in a Restek Pinnacle II C18 5 µm 100 mm × 2.1 mm under isocratic condition with a mobile phase comprising 20 mmol/L NH4Ac + 0.1% FoA/Methanol 30:70 (v/v) at a flux of 0.2 mL/min. The injected volume was 10 µL. Chromatographic apparatus (with the exclusion of the auxiliary pump and the switching valve) and mass spectrometer equipment and settings were the same as for the on-line SPE LC-MS/MS procedure.

### Statistical analysis

Data are expressed as the average ± SD. Agreement between methods was assessed by means of the Passing-Bablok robust regression, with the scat terplot of perpendicular residuals to show individual differences. Particularly, the Anderson-Darling test was used to assess the normality of residuals, and 2.5th and 97.5th percentiles were calculated. Thereby, residuals lying outside the 2.5th–97.5th interval
addressed samples for which methods poorly agreed in a significant way. The 90% confidence interval (90% CI) method was used to assess equivalence of methods with respect to the AUC measurement (22). Briefly, equivalence is stated whenever the 90% CI around the average difference δ between two methods completely lies within an equivalence interval corresponding to a certain bias percentage. In this case, the equivalence interval was set at ±5% bias of reference method.

Statistical significance was assessed for P<0.05. All calculations were done with MedCalc 12.2.1.0 (MedCalc Software bvba, Ostend, Belgium), except for TOST performed with Minitab 17.1.0 (Minitab Inc., State College, PA). Bu AUC was calculated using the trapezoidal rule with a custom Microsoft Excel electronic spreadsheet (busfile.xls available on request).

Results

The retention time measured by repeated injections was 1.58±0.003 minutes for Bu and 1.58±0.005 minutes for D8-Bu, with a 4 minutes total run time. No appreciable matrix effect was shown when tested through the post-column infusion method. Chromatograms for BM and a real sample are shown in Figure 1.

The assay was shown to be linear in the 37.75–2,416 ng/mL range against a non-linear quadratic (F-test P=0.87) and cubic fitting (F-test P=0.25). Hence, the seven-point calibration curve weighted 1/X showed r²>0.999, returning b=7.65×10⁻⁴ with SDR=1.51×10⁻⁵. Thus, the estimated LOD and LLOQ were 6.51 ng/mL and 19.74 ng/mL respectively. Particularly, a quintuplicate analysis of a Bu-free plasma sample spiked with Bu at 20 ng/mL showed CV=10.5% (Figure 2), which resulted acceptable at a value close to the LLOQ (19). No significant carryover was shown, with a pre-to-post reinjection average difference of 0.45% and 0.25% for QC3 and BM respectively.

![Figure 1](image1.png)

**Figure 1** Extracted chromatograms of Bu (A) and D8-Bu (B) in a BM sample, and of Bu (C) and D8-Bu in a real sample with plasmatic Bu concentration of 297.53 ng/mL; the mass transitions are 264.1→151.1 m/z and 272.1→159.1 m/z for Bu and D8-Bu respectively.

![Figure 2](image2.png)

**Figure 2** Extracted ion chromatogram (264.1→151.1 m/z mass transition) of a blank pooled plasma sample spiked with Bu at 20 ng/mL showing the chromatographic peak near to the estimated LLOQ of 19.74 ng/mL.
Table III Precision (%RSD), accuracy (%RE) and recovery (%±SD) of plasmatic Bu assay by on-line SPE-LC-MS/MS.

| Nominal Bu (ng/mL) | %RSD  | %RE  | Recovery (%±SD) | %RSD  | %RE  | %RSD  | %RE  | %RSD  | %RE  | %RSD  | %RE  | %RSD  | %RE
|-------------------|-------|------|----------------|-------|------|-------|------|-------|------|-------|------|-------|------
| 310               | 2.85  | -3.84| 97.1±4.5       | 2.51  | -3.97| 0.70  | -4.03| 2.40  | -5.16| 0.52  | -6.18| 1.02  | -4.72|
| 620               | 1.69  | 2.35 | 100.2±5.8      | 1.72  | 2.69 | 1.20  | 2.86 | 2.16  | 0.79 | 1.38  | -0.33| 1.36  | 1.67 |
| 1,240             | 1.27  | 0.20 | 100.9±7.6      | 1.52  | 0.15 | 2.02  | 0.06 | 1.02  | -2.69| 0.49  | -3.97| 1.98  | -1.25|
| Slope (b)         | 7.63×10^{-4} |    | 7.67×10^{-4} | 7.74×10^{-4} | 7.86×10^{-4} | 8.03×10^{-4} |    | 7.36×10^{-4} |    | 7.50×10^{-4} |    | 7.63×10^{-4} |    |
| Intercept (y)     | 4.56×10^{-3} |    | 3.03×10^{-3} | 2.67×10^{-3} | 2.18×10^{-3} | 4.18×10^{-3} |    | 3.99×10^{-3} |    | 3.61×10^{-3} |    | 4.43×10^{-3} |    |
| r²                | 0.9998 |    | 0.9998 | 0.9996 | 0.9994 | 0.9992 |    | 0.9998 |    | 0.9998 |    | 0.9998 |    |

Figure 3 A) Passing-Bablok regression plot that shows the regression curve (bold solid line) with its 95% CI (dashed line), and the identity line (dotted line) of the plasmatic Bu concentration; B) residuals plot showing the no-bias line (dashed-dotted line) and the 2.5th–97.5th percentiles range (dashed line); solid arrows (numbered 1 to 4) indicate samples with significant individual bias.
Within-day accuracy ranged between –4.72% and 1.67%, while regarding precision the intra-run RSD was between 2.85% and 0.52% and the inter-run RSD between 1.02% and 1.98%. Recovery at three Bu levels (QC1, QC2, QC3) ranged between 97.1±4.5% and 100.9±7.6%. The results are detailed in Table III.

The Passing-Bablok regression analysis returned a 0.99 slope (95% CI: 0.97 to 1.01) and –6.82 intercept (95% CI: –15.23 to 3.53) (Figure 3A). Thus, the new assay with on-line SPE produced no statistically significant bias in the Bu analysis compared to the previous method. Notwithstanding, four samples encroached the 2.5th–97.5th percentiles interval (–84.66 ng/mL to 47.83 ng/mL respectively, Figure 3B), and thus were further investigated because of significant individual bias. Particularly, samples identified as 1 and 2 gave an overestimated measure by the on-line SPE method, whilst 3 and 4 gave an underestimation.

The average value for 20 individual daily AUC was 984.9±179.2 μmol/L*min and 1015.2±190.2 μmol/L*min for the off-line SPE and the on-line SPE method respectively, with an average difference d=27.6±60.4 μmol/L*min. The ±5% bias for equivalence with respect to the off-line SPE method equated ±49.2 μmol/L*min; thereby, the two methods resulted equivalent within the given interval (90% CI of d: 9.6 to 45.5 μmol/L*min) (Figure 4).

### Discussion

In a modern clinical laboratory, where LC-MS/MS is burdened by the TDM of many drugs, as well as the assay of some other analytes (e.g. catecholamines, vitamin D), the analytical runtime must be as short as possible to make the instruments readily available (23). Of course, the duty time of laboratory personnel should also be short enough so that the automating process becomes mandatory. Indeed, our past daily routine for the TDM of Bu sampled with a 4-point LSS was carried out reserving a single LC-MS/MS apparatus during the entire duration of the procedure, which took four consecutive morning shifts (14, 21, 24). Afterwards, due to a change of the costs management, a single LC-MS/MS system was devoted to running all the TDM assays the laboratory was in charge of, so that we had to adapt the previous method to this new situation. Foremost, we maintained the isocratic separation that allowed us to shorten the dead time between injections, using a pre-mixed mobile phase on a single liquid channel of the chromatographic apparatus. Then, we adopted the on-line SPE that allowed us to free the laboratory personnel from the burden of a cumbersome sample treatment. Noteworthy, the analytical performance we obtained was comparable with that presented by other validated methods with direct injection, off-line extraction and gradient elution (9, 25).

Thus, the new method allowed us to prepare samples and condition the instrument within 5 minutes, spending about 30 minutes more to carry out a 3-point quality control and assay 4 samples. Hence, it returned the complete daily AUC within 40 minutes on average, almost as much time as is usually spent to extract Bu from plasma, or that is needed to carry out the analysis of just 4 real samples with other methods (23, 25). Noteworthy, the change of method did not cause any significant effect on the calculated AUC, with equivalent results within a bias as low as ±5% that means no significant impact on the clinical decision making for Bu dosing adjustment. Therefore, what we have presented is suitable for ordinary HPLC equipment with a switching valve, offering the possibility to reach an intermediate throughput (up to 14 samples/hour) without any newer and expensive device (26).

In establishing this assay, we aimed at robustness, preventing instrumental downtime and improving reliability. Of course, shortening the sample preparation might appear an issue for the lifetime of the chromatographic column, which is the most expensive consumable item in an LC-MS/MS assay. Actually, by means of the on-line SPE LC-MS/MS method we performed almost as much real samples as we usually did in our past routine, reaching about 250 injections before observing any backpressure increase or retention time drifting. In this regard, it is remarkable that adopting a counter-flow elution step
in respect to the SPE loading allowed to run no more than 30 samples before the system stopped due to excessive backpressure. Noteworthy, the off-line PP played a role in maintaining the whole system functionality, although this resulted in slowing the overall procedure compared to other CST-based methods running without off-line PP (27).

In this paper, we used the analysis of residuals to find sample producing individual disagreement between methods. Such an approach was based on the idea formerly advanced by Eksborg, even though nowadays it is known under the formulation proposed by Bland and Altman through their scatterplot method (28, 29). Differently, in our approach we used the residuals (deviation from actual point of the regression line) produced by the methods agreement regression, plotted by the Bu concentration predicted by the same model. To this end, we applied a 2.5th–97.5th percentiles range, defining as significantly biased the ones encroaching the 95% boundaries around the no-bias line. It should be noticed that the Bland-Altman plot used the simple difference between the results produced by new and old methods, neglecting to account for the effect of trend in data. Instead, we used residuals returned by the regression, which are detrended values suitable for a horizontal plot with parallel ranges (30). Thus, samples marked »1« and »2« in Figure 2B showed significant overestimation (positive residual) by the new method, so that we investigated carryover as a potential bias through regression residuals analysis. Noteworthy, some of the samples from this patient presented fine clumps after being thawed for a second Bu assessment a few weeks later, whose presence had not been previously reported. Thus, maybe such clumps were present but unnoticed at the first thawing, so we speculated that they could also have interfered with the correct IS spiking in this case.

Conclusions

A simple LC-MS/MS assay based on isocratic separation and on-line SPE can be successfully used to reduce the duty time of the Bu assay, allowing to complete a whole 4-point AUC analysis within 20 minutes. As relying on simple isocratic separation, it uses a single pre-mixed mobile phase, and thus it can be easily implemented on any LC system running other methods. The identification of samples with potential bias through regression residuals analysis was useful to show that individual issues were not likely due to the new analytical method.

Acknowledgements. Authors thank Andrea Capitani, BSc, for his precious contribution to the method development.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

References

17. Use ICoHoTRfRoPhH. Validation of analytical procedures: text and methodology Q2(R1); 2005.

Received: October 8, 2016
Accepted: October 29, 2016