A simplified method for busulfan monitoring using dried blood spot in combination with liquid chromatography/tandem mass spectrometry

ANSARI DJABERI, Marc Georges, et al.

Abstract

Busulfan (Bu) is an important component of the myeloablative conditioning regimen prior to hematopoietic stem cell transplantation (HSCT) especially in children. Intravenously administered Bu exhibits a therapeutic window phenomenon requiring therapeutic drug monitoring. Analytical methods developed for Bu routine monitoring were aimed at using low volumes of biological fluids and development of simple procedures to facilitate the dosage adjustment. In this report, we describe a simple, rapid method for Bu measurement using dried blood spots (DBS) from only 5μL of whole blood.

Reference


DOI : 10.1002/rcm.6241
PMID : 22592987
A simplified method for busulfan monitoring using dried blood spot in combination with liquid chromatography/tandem mass spectrometry

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4Clinical Pharmacology and Toxicology Service, Geneva University Hospitals, Geneva, Switzerland

RATIONALE: Busulfan (Bu) is an important component of the myeloablative conditioning regimen prior to hematopoietic stem cell transplantation (HSCT) especially in children. Intravenously administered Bu exhibits a therapeutic window requiring therapeutic drug monitoring. Analytical methods developed for Bu routine monitoring were aimed at using low volumes of biological fluids and development of simple procedures to facilitate the dosage adjustment. In this report, we describe a simple, rapid method for Bu measurement using dried blood spots (DBS) from only 5 μL of whole blood.

METHODS: Bu extracted from DBS with methanol was measured by high-performance liquid chromatography with electrospray ionization and tandem mass spectrometry in multiple reaction monitoring mode using D8-Bu as an internal standard. The method was in-house validated evaluating trueness, repeatability, within-laboratory reproducibility, specificity and the lower limit of quantification (LLOQ).

RESULTS: The method was linear in the calibration range of 100–2000 ng mL$^{-1}$ ($r^2 > 0.99$) encompassing the therapeutic concentrations of Bu. A good trueness ($< 14\%$), precision ($< 10\%$), and recovery ($100\%$) were observed during validation of the method with quality controls of 300, 600 and 1400 ng mL$^{-1}$. The LLOQ was determined as 50 ng mL$^{-1}$ and no matrix or carryover effects were observed. The validated method was applied to measure Bu levels in four children receiving infusion of Bu prior to HSCT. A good correlation was observed between the Bu levels measured by DBS and dried plasma spot (DPS) ($r^2 = 0.96$) and between DPS and the GC/MS method ($r^2 = 0.92$). Bu was found to be stable in DBS up to 6 h at room temperature and for 24 h at 4°C.

CONCLUSIONS: The new DBS method facilitates earlier dosage adjustment during Bu therapy by its specific and simple procedure using 5 μL of whole blood. Copyright © 2012 John Wiley & Sons, Ltd.

Busulfan (Bu), a bi-functional alkylating agent (Fig. 1), is used as a component of myeloablative and reduced intensity regimens during hematopoietic stem cell transplantation (HSCT). Bu use is preferred in children as an alternative to total body irradiation (TBI) to avoid the serious side effects of TBI (growth retardation, cataract, altered endocrinological functions, and secondary cancers).[11–17] Bu developed as an oral dosage form and exhibits wide inter- and intra-individual variability in plasma levels when administered orally.[8,9] Recently, an intravenous (IV) formulation of Bu has been developed and consequently gained popularity, especially in children, as pharmacokinetics is more predictable after IV administration compared to the oral route. Nevertheless, important inter-individual variability still persists.[10–14] Studies have shown the association of transplantation outcomes and occurrence of adverse events with Bu plasma levels.[15–17] Bu is often administered (dose range: 0.8 to 1.2 mg kg$^{-1}$) as a continuous infusion lasting for 2 h every 6 h for 16 doses to achieve the target plasma area under the curve (AUC) range of 900–1500 μmol min L$^{-1}$ which is considered as the optimal level.[10,11,18–20] Thus, Bu exhibits a therapeutic window phenomenon, requiring adjustment of its dosage based on the monitoring of the pharmacokinetic parameters after the first dose.[20–22] Several analytical methods have been developed for the measurement of Bu levels in plasma/serum. Gas chromatography with electron capture detection or mass fragmentation was developed first to measure Bu plasma levels, which is widely used for Bu determination.[23,24] Gas chromatography coupled with mass spectrometry (GC/MS) is a sensitive method, but tedious and time-consuming because of the derivatization procedures. To facilitate the plasma concentration

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‡Correction added on September 4, 2012, after first online publication: Jules Desmules was changed to Jules Desmeules.
monitoring with ease, liquid chromatography methods have been developed using ultraviolet detection. However, Bu is not UV visible and hence derivatization is needed for this method. Development of liquid chromatography coupled to (tandem) mass spectrometry (LC/MS, LC/MS/MS) allowed rapid Bu plasma level monitoring without derivatization procedures using relatively small amounts of plasma and other biological fluids like saliva. Recently, a simple LC/MS method for rapid measurement of Bu from plasma and serum has been described using a turbulent flow online extraction procedure. Simple measurement techniques such as immunoassay and automated photometric methods have also been developed to obtain quick and reliable results with good accuracy and precision comparable to the GC/MS method. Unlike GC/MS, LC, LC/MS/MS methods, immunoassay methods for Bu determination are not widely studied for their clinical utility.

The dried blood spot (DBS) sampling method has received attention from several analytical chemists and clinicians because it offers convenient sampling and handling for pharmaceutical and biochemical analysis. The sampling procedure is less invasive and is cost effective in terms of sample collection, storage, time for analysis and management. This method also offers minimal risk of infection by pathogens. The DBS procedure has been successfully implicated in the monitoring of various therapeutic agents like tacrolimus, metformin, topiramate, ritonavir, mycophenolate and oseltamivir. It has also been used for pharmacokinetic and toxicokinetic studies. As a DBS is easy to collect and store, it can be a convenient alternative to plasma in settings with limited laboratory capacity or when collection of samples at various time points after the drug administration is required. As collection of large volumes of the blood samples for analysis could be a problem in children and infants, DBS may offer greater utility compared to conventional plasma analysis because of the very low sample it uses. It is also convenient for storage and shipment of samples collected at different centers in a clinical study and needed to be transported to the analytical laboratory. Hence, we evaluated the possibility of using DBS followed by measurement with LC/MS/MS for routine therapeutic monitoring of Bu.

**EXPERIMENTAL**

**Chemicals and reagents**

Bu and ammonium formate were purchased from Sigma-Aldrich (Steinhem, Germany). D8-Bu (Toronto Research Chemicals Inc., Ontario, Canada) was used as internal standard. HPLC grade methanol and acetonitrile were procured from Merck (Darmstadt, Germany). Formic acid was procured from Honeywell Riedel-de haen (Hanover, Germany). In vitro diagnostic 903 grade blood spot cards were purchased from Whatman (Whatman 903™ GE Healthcare, Dassel, Germany). Autosampler glass vials (0.3 mL capacity) were procured from BGB Analytik AG (Switzerland). Drug-free plasma and drug-free human blood with EDTA as anticoagulant were provided by the Geneva University Hospitals (Geneva, Switzerland).

A stock solution of Bu was prepared at a concentration of 1 mg mL$^{-1}$ in methanol. Working standard solutions were prepared by diluting stock solution in MilliQ water (Millipore, France) to reach concentrations ranging from 1 to 100 μg mL$^{-1}$. Stock solutions and working standards were prepared once every 30 days and once in 2 weeks, respectively. After use, stock solutions and working solutions were stored at $-20^\circ$C. The internal standard D8-Bu was diluted in methanol to 1 mg mL$^{-1}$ and a working solution at 2500 ng mL$^{-1}$ in methanol was used. This working solution was added to methanol used for extraction to obtain a final concentration of 100 ng mL$^{-1}$.

**Calibration standards and quality controls**

Calibration standards and quality control (QC) samples were prepared by spiking whole blood with working solutions of Bu to reach a volume of 100 μL of blood. Whole blood samples from five subjects not receiving the test drug were pooled and used to prepare Bu standards and QC samples. Calibrators were prepared in the range of 100–2000 ng mL$^{-1}$ by adding working solutions to the whole blood. Similarly three QC samples were prepared independently at concentrations of 300, 600 and 1400 ng mL$^{-1}$. Calibration standards in the range of 100 to 2000 ng mL$^{-1}$ and QC samples (300, 600 and 1400 ng mL$^{-1}$) were also prepared in pooled drug-free plasma. This attempted to compare the levels measured by both dry plasma spots (DPS) and DBS.

**Sample preparation**

Blood and plasma calibrator standards, QC samples and clinical samples (5 μL) were spotted onto Whatman 903 DBS cards using a volumetric micropipette (Eppendorf, Hamburg, Germany) and left to dry at room temperature for a minimum of 30 min. Circular discs of 6 mm diameter covering the entire spots were cut and placed into individual autosampler vials of 0.3 mL capacity. Methanol (100 μL) containing the internal standard D8-Bu at 100 ng mL$^{-1}$ was added to each vial, sealed to prevent evaporation, and vortexed for 1 min. Then, 5 μL were injected into the LC/MS/MS system.

**LC/MS equipment and conditions**

All experiments were performed using an API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada) controlled by Analyst 1.5.1 software. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with positive ion electrospray ionization. The MRM transitions were 264.1 $\rightarrow$ 151.1, and 272.1 $\rightarrow$ 159.1, with a dwell time of 150 ms for Bu and internal standard Bu-D8 (IS), respectively. Nitrogen was used as the curtain and nebulizer gas. The instrument conditions were set at: capillary
Dried blood spot sampling for busulfan plasma level monitoring

Validation procedure

The validation procedure was carried out during three non-consecutive days according to the guidelines of the “Societe Francaise des Sciences et des Techniques Pharmaceutiques”. The DBS calibration (4 concentrations) and the QC samples (3 concentrations) were prepared on each validation day in triplicates (n = 3). Samples were prepared and analyzed on three different validation days. The peak area ratios of Bu (analyte) to that of D8-Bu (internal standard) were used for all calculations. Accuracy and precision of the method for the selected QC concentration levels were determined using analysis of variance (ANOVA). The calibrators and QC samples were chosen in the defined range to cover the targeted Bu plasma levels measured during the routine monitoring.

Ion suppression and matrix effect

Ion suppression was evaluated by continuous post-column infusion of Bu (100 ng mL⁻¹) in methanol directly into the mass spectrometer via a Hamilton syringe at a flow rate of 10 μL min⁻¹ and by injecting blank DBS extracts. Matrix effect was evaluated by comparing the peak area ratios (Bu/D8-Bu) obtained after injecting the extracted blank DBS with Bu added before injection and equivalent solution of Bu in methanol. This experiment was performed at three QC concentrations, i.e. 300, 600 and 1400 ng mL⁻¹, and six independent extractions or solutions were injected for each concentration tested.

Precision, trueness and limit of detection

The trueness and precision were determined with the QC samples by recalculating the concentration with the daily response function established. Trueness was expressed as the ratio of the measured and theoretical concentrations. Precision was expressed as the relative standard deviation (RSD) of the ratio of the intra-day variance (intra-assay precision) and inter-day variance (inter-assay precision) on the theoretical value at each concentration level. The lowest concentration of the calibration curve was evaluated to be in the range of the validation guidelines (coefficient of variation (CV) < 20%). The lower limit of quantification (LLOQ) was set at 10 times the signal-to-noise (S/N) ratio.

Recovery and carryover

Recovery for Bu from DBS was assessed at three QC concentrations (300, 600 and 1400 ng mL⁻¹) by comparing the absolute peak areas of Bu obtained after injecting the DBS prepared from spiked blood and the extracted blank DBS with equivalent amount of Bu added before injection. No recovery studies for internal standard were considered, since internal standard was added to methanol used for extraction. The experiment was performed with six independent extractions for each concentration tested.

Carryover effect was investigated by analysis of spiked QC samples (300 and 1400 ng mL⁻¹) run in sequence of 300-1400-300, in triplicates. Another procedure involved running of blank samples before and after running a QC sample of highest analyte concentration (blank-1400-blank) in triplicates. Alternatively, we also compared the absolute analyte peak areas of two calibrators (High concentration and Low concentration) run in the sequence of Low-High-Low. Passing the carryover test was defined as the post-run value falling within 3 standard deviations of pre-run or detection of no peaks above the noise level in the blank samples run after high concentration QC sample.

Stability

The short-term stability of DBS and DPS was determined by preparing the spots from the three QC samples in duplicates. They were extracted and analyzed on the day of preparation and remaining DBS were stored at room temperature, 4°C and –20°C. The DBS were analyzed again with freshly prepared calibrators and QC samples after 1, 7, 14 days and 1 month. The results are expressed as the percentage ratios of the measured and theoretical concentrations. The stability of Bu was also assessed by allowing the DBS to dry at room temperature for up to 6 h. This was performed to understand the influence of drying time on the measured concentrations during the sampling procedure which lasts for about 6 h during Bu infusion.

Clinical application of DBS

Clinical samples were obtained from four patients who underwent HSCT in the Hematology and Oncology Unit of the Pediatrics Department, Geneva University Hospitals. The study protocol was approved by the Hospital Ethics Committee and patient samples were used after obtaining informed consent. The study was conducted according to the revised Declarations of Helsinki, the standards of Good Clinical Practice, and the Swiss regulatory requirements. The DPS and DBS were prepared from four patients collected at different time points (n = 44) during administration of Bu. The levels measured were compared both among themselves and with those (n = 13) obtained by the conventional GC/MS method.

The venous blood samples were collected at 0, 15, 30, 60, and 240 min after the infusion of Bu for routine therapeutic drug monitoring after dose 1, 2, 3, 5, 7 and 9. Five μL of the whole blood were directly spotted on the paper to prepare...
DBS. Plasma was separated after centrifugation (900 g for 15 min) and 5 μL was spotted on the paper. The remaining plasma was used for Bu determination by the conventional GC/MS method as previously described.[24]

RESULTS AND DISCUSSION

Validation of the method

The analytical cycle time was 7.0 min per injection. The retention times for Bu and D8-Bu (mean ± SD) under optimal conditions were 2.90 ± 0.4 and 2.91 ± 0.3, respectively (Fig. 2). No significant ion suppression and matrix effects were observed. The method was selective with no interfering peaks observed during the retention times of the analyte, which was evaluated by running the blank samples, prepared using the individual and pooled blood from subjects not receiving Bu.

In this report, we showed that a simple sampling procedure with DBS or DPS followed by a rapid extraction procedure with methanol and injection for LC/MS/MS analysis produce reliable results similar to the conventional GC/MS procedure. Previously, an online extraction procedure using an inox cell or an autosampler was proposed for DBS extraction.[43,44] Offline extraction procedures described previously were known to involve the extraction of DBS paper with solvent, followed by centrifugation and then injection into the LC/MS/MS system.[37,45] In this report, we showed that addition of extraction solvent to DBS/DPS placed in an autosampler vial followed by brief vortexing and injecting into the LC/MS/MS system allows rapid extraction and analysis. Thus, the DBS method offers accurate measurements of Bu levels using small volumes of blood without additional procedures (like plasma separation, liquid extraction) and in a short period of time. The DBS method could facilitate Bu dosage adjustment with rapid turnaround time from an analytical chemist to the clinician.

The DBS method validation was performed for precision, trueness, and recovery. Assay calibration was performed at four different levels. Quantification was performed based on the peak area ratios of Bu to D8-Bu. The most suitable response function was selected by testing different regression models (Table 1). This was determined by calculating the existing relationship between the expected concentration and the analytical response, and by applying different weighting factors. Best results were observed for a linear regression with a weighting factor of 1/x. Linearity was calculated by fitting the back-calculated concentrations of QC samples as a function of the introduced concentrations and by applying the linear regression model based on the least-squares method.[46] Assay was found to be linear in the tested concentration range and the lowest concentration used in the calibration curve (100 ng mL⁻¹) had a precision of <10% and a trueness within 10% (Table 1). Thus the lowest concentration of the calibration curve is quantifiable. Bu is administered by infusion and measured levels are usually above 100 ng mL⁻¹; hence, the present calibration range is acceptable for measuring the levels of Bu for routine monitoring.[19] The QC samples were also chosen to encompass the clinically relevant levels of Bu after intravenous infusion (1 mg kg⁻¹ for 2 h).[11,20] The lower limit of quantification (LLOQ) was determined as 50 ng mL⁻¹ (signal-to-noise (S/N) ratio = 10).

The trueness over the three concentration ranges tested was between 5.2% and 13.8% (Table 2). The precision calculated as intra-assay precision and inter-assay precision values were below ±10% for each QC concentration tested (Table 2). The recovery of Bu was determined to be equal to or more than 100% for the QC samples tested (Table 2). The peak area ratios obtained from DPS and DBS for the same calibrator and QC samples were found to be similar (<20% difference) suggesting no influence of cellular portion or hematocrit on recovery (data not shown). No carryover was observed when a low QC sample (300 ng mL⁻¹) was run before (mean ± SD = 314.7...
Bu in plasma is stable up to 24 h at 4°C and whole blood, stability of Bu in DBS is not known. Even though studies have reported the stability of Bu in storage and shipment conditions for Bu DBS and DPS papers. However, Bu is stable up to 12 h at room temperature when extracted and re-dissolved in mobile phase. Bu was also reported to be stable in whole blood up to 24 h at 4°C and in plasma was found to be stable up to 2 years when stored at −80°C. Bu in test solutions was reported to be stable for 8 h when stored in glass bottles at 20°C. In aqueous media, Bu rapidly hydrolyzes to tetrahydrofuran and methanesulfonic acid in a concentration-dependent manner and is highly dependent on temperature. Bu reacts with nucleophiles by an SN2 mechanism. The binding ability of Bu to the macromolecular components of blood cells also increases upon storage which may enhance its degradation.

The short-term stability was investigated at three temperatures (ambient, 4°C and −20°C) over 30 days to evaluate the storage and shipment conditions for Bu DBS and DPS papers. Even though studies have reported the stability of Bu in plasma and whole blood, stability of Bu in DBS is not known. Bu in plasma is stable up to 24 h at 4°C and more than 75% degradation occurred within 24 h when kept at room temperature. However, Bu is stable up to 12 h at room temperature when extracted and re-dissolved in mobile phase. Bu was also reported to be stable in whole blood up to 24 h at 4°C and in plasma was found to be stable up to 2 years when stored at −80°C. Bu in test solutions was reported to be stable for 8 h when stored in glass bottles at 20°C. In aqueous media, Bu rapidly hydrolyzes to tetrahydrofuran and methanesulfonic acid in a concentration-dependent manner and is highly dependent on temperature.

### Table 1. Intra and inter assay precision and accuracy in calibration for busulfan method validation using DBS sampling and respective linear regression data

<table>
<thead>
<tr>
<th>Calibrator sample</th>
<th>Assay</th>
<th>Concentration (ng mL⁻¹)</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
<th>Inter-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD (n = 3)</td>
<td>trueness (%)</td>
<td>precision</td>
<td>trueness</td>
<td>precision</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>109.1 ± 10.8</td>
<td>109.1</td>
<td>9.9</td>
<td>109.0</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>109.0 ± 1.4</td>
<td>108.9</td>
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<td>108.8</td>
<td>1.3</td>
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<tr>
<td></td>
<td>3</td>
<td>109.1 ± 10.8</td>
<td>109.1</td>
<td>9.9</td>
<td>109.0</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>199.4 ± 5.9</td>
<td>99.7</td>
<td>2.9</td>
<td>101.9</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>212.9 ± 31.1</td>
<td>106.4</td>
<td>14.6</td>
<td>106.5</td>
<td>14.9</td>
</tr>
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<td></td>
<td>3</td>
<td>199.4 ± 5.9</td>
<td>99.7</td>
<td>2.9</td>
<td>101.9</td>
<td>6.8</td>
</tr>
<tr>
<td>1000</td>
<td>1</td>
<td>983.8 ± 48.0</td>
<td>98.4</td>
<td>4.9</td>
<td>97.6</td>
<td>4.3</td>
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<tr>
<td></td>
<td>2</td>
<td>959.7 ± 31.0</td>
<td>95.9</td>
<td>3.2</td>
<td>95.9</td>
<td>3.2</td>
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<tr>
<td></td>
<td>3</td>
<td>983.8 ± 48.0</td>
<td>98.3</td>
<td>4.9</td>
<td>97.6</td>
<td>4.3</td>
</tr>
<tr>
<td>2000</td>
<td>1</td>
<td>2007.7 ± 69.6</td>
<td>100.4</td>
<td>3.5</td>
<td>100.6</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2018.4 ± 37.9</td>
<td>100.9</td>
<td>1.9</td>
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<td>1.9</td>
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<td>3</td>
<td>2007.7 ± 69.6</td>
<td>100.4</td>
<td>3.5</td>
<td>100.6</td>
<td>3.0</td>
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</table>

Regression equations after 1/x weighting

<table>
<thead>
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<th></th>
<th>Assay</th>
<th>Regression equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y = 0.0019X + 0.0011</td>
<td>r² = 0.9993</td>
</tr>
<tr>
<td>2</td>
<td>Y = 0.0020X + 0.0016</td>
<td>r² = 0.9986</td>
</tr>
<tr>
<td>3</td>
<td>Y = 0.0021X + 0.0039</td>
<td>r² = 0.9990</td>
</tr>
</tbody>
</table>

### Table 2. Validation summary for busulfan determination by dried blood spot sampling followed by LC/MS/MS

<table>
<thead>
<tr>
<th>Nominal concentration (ng mL⁻¹)</th>
<th>Assay</th>
<th>Measured concentration mean ± SD (n = 4)</th>
<th>Intra-assay precision (RSD %)</th>
<th>Inter-assay precision (RSD %)</th>
<th>Inter-assay trueness (%)</th>
<th>Recovery mean ± SD (n = 6)</th>
<th>Matrix effect (% mean ± SD (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>Day 1</td>
<td>351.9 ± 3.2</td>
<td>4.4</td>
<td>5.8</td>
<td>113.8</td>
<td>116.6 ± 9.6</td>
<td>108.17 ± 9.7</td>
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<tr>
<td></td>
<td>Day 2</td>
<td>348.5 ± 5.5</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>318.1 ± 16.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>Day 1</td>
<td>675.8 ± 29.6</td>
<td>6.2</td>
<td>9.7</td>
<td>105.2</td>
<td>118.4 ± 11.9</td>
<td>101.8 ± 25.2</td>
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<tr>
<td></td>
<td>Day 2</td>
<td>634.4 ± 16.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>567.1 ± 15.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1400</td>
<td>Day 1</td>
<td>1620.9 ± 33.0</td>
<td>7.8</td>
<td>7.8</td>
<td>111.7</td>
<td>101.03 ± 11.7</td>
<td>99.3 ± 11.3</td>
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<tr>
<td></td>
<td>Day 2</td>
<td>1539.1 ± 32.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>1519.6 ± 228.1</td>
<td></td>
<td></td>
<td></td>
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</table>
Table 3. Stability test of dried blood spot (DBS) and dried plasma spot (DPS) samples for busulfan determination

<table>
<thead>
<tr>
<th>Storage</th>
<th>Concentration</th>
<th>24 h</th>
<th>1 week</th>
<th>2 weeks</th>
<th>1 month</th>
<th>24 h</th>
<th>1st week</th>
<th>2nd week</th>
<th>1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient room</td>
<td>300</td>
<td>45.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>56.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>temperature</td>
<td>600</td>
<td>45.2</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>69.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>+4°C</td>
<td>1400</td>
<td>46.4</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>67.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>100.0</td>
<td>80.0</td>
<td>55.3</td>
<td>30.6</td>
<td>100.0</td>
<td>87.0</td>
<td>29.0</td>
<td>26.0</td>
</tr>
<tr>
<td>−20°C</td>
<td>600</td>
<td>110.0</td>
<td>74.5</td>
<td>58.7</td>
<td>28.6</td>
<td>114.0</td>
<td>95.9</td>
<td>33.0</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>1400</td>
<td>101.0</td>
<td>80.5</td>
<td>62.2</td>
<td>22.4</td>
<td>105.0</td>
<td>88.7</td>
<td>38.0</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>–</td>
<td>100.9</td>
<td>100.9</td>
<td>95.0</td>
<td>–</td>
<td>101.3</td>
<td>96.0</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>–</td>
<td>99.3</td>
<td>100.4</td>
<td>89.0</td>
<td>–</td>
<td>101.0</td>
<td>91.0</td>
<td>93.0</td>
</tr>
<tr>
<td></td>
<td>1400</td>
<td>–</td>
<td>114.4</td>
<td>108.8</td>
<td>100.2</td>
<td>–</td>
<td>109.0</td>
<td>110.0</td>
<td>91.0</td>
</tr>
</tbody>
</table>

The measurements were not performed at 24 h period for DBS kept at −20°C based on previous reports on its stability.

for up to 6 h (Fig. 3). However, the data showed that Bu in both DBS and DPS was not stable when stored at ambient room temperature for 24 h, suggesting that DBS or DPS should be stored at −20°C for long periods. These results differed from the earlier reports on stability of other compounds in DBS, which suggested that samples can be stored and shipped at ambient temperature. Even though DBS offers a mechanism for providing good stability to a few unstable compounds, some compounds were reported as unstable in DBS when stored at room temperature. Studies have shown that usage of a chemically modified DBS paper or derivatization procedures might improve the stability of the compounds in DBS paper. However, the chemical treatment procedure to improve the stability of Bu was not evaluated in the present study. The stability issue may not impede the routine therapeutic monitoring, since Bu was found to be stable up to 6 h at room temperature. However, low sample volume, simplified procedure, avoiding venous blood sample collection with true capillary DBS sampling, low storage space and rapid analysis by DBS method must outweigh the stability issue of Bu in DBS for routine monitoring of Bu levels.

Clinical application of DBS

All the samples were collected from four children undergoing Bu infusion before hematopoietic stem cell transplantation and were analyzed on the same day as mentioned in the Experimental section. The venous blood was collected in EDTA tubes at 0, 15 min, 30 min, 1 h and 4 h after the end of Bu infusion at different dose times (1, 2, 3, 5, 7 and 9). Bu was measured using a DBS paper spotted with venous blood collected with EDTA as anticoagulant and DPS spotted with plasma separated from the same whole blood sample (n = 44). Furthermore, Bu measurements were taken using a conventional GC/MS method (n = 13) from a few plasma samples of one child. This was performed to correlate the blood concentrations with that of plasma and to explore the utility of this method for routine monitoring of Bu.

A good correlation was observed between the levels measured by DBS and DPS with a linear correlation coefficient of (r²) 0.96 (Fig. 4). The Bu levels measured by DBS were lower compared to levels measured by DPS (slope = 0.89) (Fig. 5(A)). The Bu levels measured by DPS were also correlated with those measured by a conventional GC/MS method and were within the acceptable range (< ±15%; r² = 0.92; slope = 0.95; Fig. 5(B)). The levels estimated by DBS were less than those obtained by DPS and GC/MS methods (Figs. 5(A) and 5(B)). The hematocrit (Hct) values in the four patients were in the range 25.6 to 30.3% which could explain the observed differences between DBS and DPS Bu levels. The Hct value must be considered when whole blood is used for analysis, particularly when a specific area of the DBS is punched, because the viscosity, volume and distribution of blood on the paper could be altered by its cellular
Correlation of busulfan levels measured by dried blood spot (DPS) and dried blood spot (DBS) sampling from two patients followed by LC/MS/MS analysis. Bu levels at different dose times (1, 2, 3, 5, 7 and 9) were measured using DBS and DPS. DBS and DPS were prepared from the venous blood samples collected at each dose level during the entire five different time points or any two time points, i.e. one at the end of infusion and at 15 min, 30 min, 1 h, 4 h after the end of infusion (n= 44 samples run in duplicates). The hematocrit values of these four patients were in the range of 25.6–30.3% at different dose levels.

Figure 5. Comparison of busulfan levels measured by DBS, DPS sampling followed by LC/MS/MS and GC/MS using plasma from a patient after the first dose of busulfan. (A) The percentage difference in the busulfan levels measured by DBS sampling (circles) from DPS sampling followed by LC/MS/MS analysis. (B) The percentage difference in the busulfan levels measured by DBS sampling (triangles), DPS sampling (circles) from the levels measured from plasma by the GC/MS method.

component. An approach to reduce the impact of Hct was proposed for accurate sampling using perforated DBS or pre-cut DBS. The good correlation observed between DPS and DBS with a regression slope greater than 0.8 also suggests that the distribution of Bu may be even between plasma and blood cells. A previous study reported that up to 46% of Bu binds to red blood cells irreversibly. Therefore, a significant proportion of Bu binding to blood cells may influence the pharmacokinetic measurements of DBS Bu in subjects with very low and very high Hct levels. The observed difference between DBS and DPS levels may be due to other cellular debris in the whole blood diluting the Bu concentration compared to plasma. Moreover, the extraction procedure used (vortexing with 100 μL of methanol) may not be effective to recover overall bound fraction of Bu. Our study demonstrated a good correlation between DBS and DPS samples in pediatric patients with low Hct values, suggesting the utility of this method in these patients. Establishing usage of DBS as an alternative sampling method to plasma for therapeutic drug monitoring requires clinical validation with a large number of samples with wide ranges of Hct values.

A good correlation was observed between the levels measured by the DBS method and a conventional GC/MS method (Fig. 5(B)), with a linear correlation coefficient ($r^2$) of 0.91 and a linear regression slope of 0.80. Previous studies have shown good correlation between the levels of analyte measured in capillary and venous blood. The possibility of implementing capillary DBS sampling for therapeutic monitoring of Bu in children must be assessed in the future as it can avoid the need to collect venous blood samples. It is advisable to collect the blood sample for measurement of Bu from the peripheral intravenous line to avoid contamination from the central venous line where it is administered. Alternatively, we propose that peripheral IV cannulation can be avoided by collecting capillary blood samples from a finger prick by DBS. In most centers, the blood samples are collected from the central venous line after flushing it with saline to avoid contamination from infused Bu. In such cases, these additional procedures (finger prick) could be avoided and venous blood samples from central venous lines could be used for DBS in patients undergoing Bu infusion for routine monitoring. Figure 6 illustrates the pharmacokinetic profile of Bu in a child receiving Bu infusion (0.8 mg kg$^{-1}$) before HSCT. Bu was detected with a high signal close to the end of the infusion. This indicates that the method meets all the requirements for its utility in pharmacokinetic studies and routine therapeutic drug monitoring. Further clinical studies must evaluate the relevance of capillary blood collection by DBS for routine therapeutic drug monitoring. Implementation of the DBS sampling method after clinical validation could be of great value for measurement of Bu levels in patients on oral Bu and in pediatric patients receiving Bu intravenously.
CONCLUSIONS

In conclusion, we have developed and validated a novel method to quantify Bu by using blood or plasma spots of 5 μL only. The method is rapid, accurate and requires minimal sample preparation with rapid turnaround time for dosage adjustment. No matrix or carryover effects were observed with the DBS method. The same method could be implemented in clinical practice for routine measurement of Bu levels. However, further clinical validation of the DBS method needs to be performed for its utility in clinical studies and routine therapeutic drug monitoring of Bu.

Acknowledgements

This work was supported by the CanSearCH Foundation and the Geneva Cancer League. We thank Fabienne Doffey Lazeyras for her help in sample preparation, Virginie Ancrenaz for her help in the LC-MS/MS instrument operation, and Patricia Huezo Diaz for proof reading the manuscript.

REFERENCES


Dried blood spot sampling for busulfan plasma level monitoring


