Fast and reliable quantification of busulfan in blood plasma using two-channel liquid chromatography tandem mass spectrometry: Validation of assay performance in the presence of drug formulation excipients

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A fast and reliable method based on two-channel liquid chromatography coupled to tandem mass spectrometry was developed and successfully validated for quantification of busulfan. The drug vehicle polyethylene glycol 400 was quantified simultaneously in patient samples. The sample preparation consisted of simple protein precipitation using a mixture of methanol and zinc sulphate containing busulfan-d₈ as internal standard. Chromatographic separation was performed on a short biphenyl column (30 mm × 3.0 mm, 5 μm particles) using a step gradient from 30 % to 85 % methanol, ensuring co-elution of the analyte and internal standard. Quantification was performed using the mass transition of 264.1 > 151.1 for busulfan and 272.1 > 159.1 for the internal standard. Using only 20 μL of plasma sample, the lower limit of quantification was 25 ng/mL. Signal to noise ratio at the lower limit of quantification exceeded 300. The assay performance was not adversely affected by matrix effects originating from drug formulation excipients or other sample components. The coefficient of variation was <4 % and the mean accuracy 101–108 % across the calibration range 25–5 000 ng/mL. Chromatographic run time was 2 min and 8 s, allowing an effective run-time of 1 min and 10 s when using two alternating LC-channels. The assay has been implemented in routine practice with accreditation according to the ISO 15189 standard, and performs well in external quality control assessments. We present for the first time that shortly after an IV infusion of busulfan, the plasma levels of polyethylene glycol 400 may be in the range of 400–800 mg/L. The presence of these levels of detergent in patient samples may have detrimental effects on assay performance in LC–MS/MS, not limited to busulfan assays. This may be a concern for any LC–MS/MS analysis performed on samples collected within the first 24 h after an IV infusion of busulfan.

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1. Introduction

Following decades of use in chemotherapy, the alkylating agent busulfan is nowadays mainly used for myeloablation in preconditioning before hematopoietic stem cell transplantation (HSCT). For several years, only formulations for oral administration were available. However, during the last two decades, busulfan for intravenous use has gradually been introduced [1]. With oral use, the erratic bioavailability of busulfan was a problem which was reinforced by the emetic effect of the drug. Hepatic first pass and high plasma concentrations following oral dosing probably contributed to severe adverse effects like sinusoidal obstruction syndrome (SOS) and mucositis. In order to reduce treatment related mortality, in our center as in many others, therapeutic drug monitoring (TDM) of busulfan was regularly performed, ideally from the first day in order to prevent continued exposure to high concentrations [2]. With the introduction of intravenous (IV) busulfan one might question whether TDM would show advantage over dosing adjusted only to body weight or surface area. A large retrospective
multicenter study showed that the prognosis, both in terms of outcome and toxicity, is indeed related to an identified busulfan target exposure in context of IV drug administration [3], and a similar conclusion was presented in a recent review [4]. Such studies have also highlighted the importance of adequate methods for the bioanalysis of busulfan and for the appropriate calculation of individual pharmacokinetic characteristics as prerequisites for the successful personalization of busulfan dosing.

Dimethylacetamide (DMA) and polyethylene glycol 400 (PEG 400) are frequently used excipients in IV busulfan formulations. Each vial of 60 mg busulfan contains 3.3 mL DMA and 6.7 mL PEG 400 [5]. The toxicological aspects of IV DMA in busulfan patients have attracted some attention [6], whereas this has not been addressed for PEG 400. Another aspect relates to the potential untoward effects these excipients may have on the analytical performance when busulfan is quantified in blood plasma. The European Medicines Agency (EMA) guideline on bioanalytical method validation states that potential matrix effects caused by drug formulation excipients should be investigated as a part of the method validation [7]. A number of analytical methods for busulfan in blood plasma have been published over the last ten years [8–16], but the potential influence of DMA and PEG 400 on the analytical performance has to the best of our knowledge neither been examined nor discussed.

Of 22 analytical methods in use at TDM centers for the determination of busulfan (reviewed in [17]), five were based on gas chromatography, four were based on HPLC with UV detection, and 12 on LC–MS/MS. All of the 16 liquid chromatography methods used alkane type reversed phase columns (C-8 to C-18) for separation. Sample volumes ranged from 50 to 700 μL, one method used a 5 μL dried plasma spot. Chromatographic runtime ranged from 2 to 20 min. The time required for sample preparation or total turnaround time for a complete set of samples was not listed. Two methods reported using simple protein precipitation for sample preparation followed by a chromatographic runtime of 2 min, a combination that has a potential for short turnaround time.

The aim of this study was to develop a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the reliable quantification of busulfan in patient plasma, providing sufficient performance even in the low concentration range of pre-dose samples during once-daily IV dosing regimens. A priority was to establish an assay suitable for everyday routine use, with a fast turnaround time and using a small sample volume.

We describe here a fast and sensitive method for the determination of busulfan, using a novel separation mechanism and using only 20 μL of sample. The method also allows for the quantification of PEG 400 in patient samples in the same run. The present method was validated in accordance with the EMA and FDA guidelines, and it has been implemented in routine practice with accreditation according to the ISO 15189 standard.

2. Materials and methods

2.1. Chemicals and reagents

Busulfan (purity 99.7 %) for the preparation of calibrators and quality controls (QC) was purchased as a certified solution of 1.000 ± 0.005 mg/mL from Sigma-Aldrich (Oslo, Norway). Busulfan-d8 (purity 99 %) from C/D/N Isotopes Inc. (Quebec, Canada) was used as internal standard (IS). HPLC-grade acetonitrile and methanol were obtained from Rathburn Chemicals (Walkerburn, Scotland). Ethylenediaminetetraacetic acid (EDTA) solution 500 mmol/L, LC–MS grade ammonium acetate, HPLC-grade water, zinc sulphate 0.10 mol/L, DMA and PEG 400 solution were purchased from Sigma-Aldrich. Mobile phases were prepared with UHPLC-MS grade water and methanol with 10.0 % formic acid from Honeywell (Morris Plains, NJ). Autonorm drug-free animal serum was a product from Sero AS (Billingstad, Norway). Autonorm was dissolved as described by the supplier.

A stock solution of deuterated IS (0.05 mg/mL) was prepared by dissolving 2.5 mg of the substance in 50 mL of acetonitrile. The solution was divided into 2 mL aliquots and stored at −70 °C. One tube at the time was stored at −20 °C for up to one year, and used for the preparation of the precipitation reagent. The precipitation reagent was made up by blending 240 mL methanol and 120 mL zinc sulphate solution in a 500 mL glass bottle. The solution was cooled to +4 °C before the addition of 200 μL of IS solution. This precipitation reagent was stored at +4 °C for up to 8 weeks. The concentration of busulfan-d8 in the solution was 28 ng/mL, corresponding to approximately 700 ng/mL of IS per plasma sample.

2.2. Preparation of calibrators and quality control samples

As the stability of busulfan in plasma or serum is dependent on temperature, the preparation and aliquoting of calibrators and controls was performed as a continuous process, i.e. the time at room temperature before aliquoting and freezing at −70 °C was kept at a minimum.

The three high calibrators (5000, 2000 and 1000 ng/mL) were made up by pipetting the certified busulfan solution into three separate volumetric flasks (20 mL, 50 mL and 50 mL, respectively) using a calibrated 100 μL Hamilton syringe. The flasks were filled to volume with Autonorm. Calibrators at 667, 222, 74.1, and 24.7 ng/mL were prepared by serial threefold dilutions of the 2000 ng/mL calibrator in Autonorm. Thus, a total of seven non-zero calibrator levels and one calibrator blank (near Autonorm) were included. QCs were made up the same way from the same vial of certified busulfan solution. The high and medium QCs (4000 and 1000 ng/mL) were prepared by pipetting the certified busulfan solution into two separate volumetric flasks (25 mL and 50 mL, respectively), and filling the flasks to volume with Autonorm. The low level QC (50 ng/mL) was obtained by diluting the medium control twentyfold in Autonorm. Neat Autonorm was used as QC blank. A separate QC at the lower limit of quantification (LLOQ, 25 ng/mL) for use during the validation process, exclusively, was prepared by diluting the low QC twofold in Autonorm. All calibrators and controls were aliquoted and stored in an ultrafreezer (≤ −70 °C) until the day of use. Calibrators in 300 μL aliquots and QCs in 500 μL aliquots, both in 0.5 mL polypropylene tubes with O-ring sealed screw caps (Sarstedt # 72.730.406).

External QC: Our laboratory participated in a busulfan cross verification exercise organized by the Cansearch laboratory (Dr M. Ansari, Geneva, Switzerland) [17], and also in the external quality assessment scheme organized by the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML, https://www.ukml.nl).

Calibrators and QC for PEG 400 were made by serial dilution of stock solutions of 10 g/L of PEG 400 dissolved in Autonorm. The stock solutions were prepared by pipetting 100 mg of PEG solution into two separate 10 mL volumetric flasks (one for calibrators, and one for QCs), and filling the flasks to volume with Autonorm. Calibrator levels were 50, 30, 10, 7.5, 5.0, 3.0 and 1.0 mg/L. QC levels were 50, 25 and 2.5 mg/L. The solutions were aliquoted and stored at −70 °C until the day of use.

2.3. Samples; handling and preparation

Patient samples used for development, validation and quality assessment of the present analytical assay were either processed according to current standard practice and protocols for busulfan treatment at our institution, or anonymized surplus of such samples. No extra sample was drawn for the purpose of this project. Venous blood from patients were sampled in EDTA vacutainers
without gel, and centrifuged at 4 °C for 10 min at 2000 g. The resulting plasma was stored in a refrigerator until analysis within 24 h. Calibrators and QC s were thawed at room temperature for 15 min and thereafter kept at 4 °C for up to 48 h. One set of calibrators and QC s was used for up to two consecutive days of analysis, but for no longer than 48 h after thawing. Time at room temperature was kept at a minimum, and limited to the time of preparation of samples. On each analysis day, one fresh tube of in-house long term QC (patient pool) was thawed from ~70 °C.

Samples from patients receiving busulfan orally were prepared undiluted. Samples from patients receiving busulfan intravenously were pre-preparation diluted in Autonorm containing 5 mmol/L EDTA (before use, 50 μL of a 500 mmol/L EDTA-solution was added to each 5 mL vial of Autonorm). EDTA was used to prevent the formation of clots when diluting plasma samples. The dilutions followed a predefined protocol. The dilution scheme was customized to fit samples from patients administered busulfan intravenously as a 3 h infusion aiming for a concentration of drug at steady state (Css) of 750–900 ng/mL. A total of 8–9 samples were collected at each dose interval: Pre-dose, and then 5 min, 30 min, 1 h, 2, 3, 5, 7, and 10 h after end of infusion. Sample 1 (pre-dose), 8 and 9 was diluted twofold (200 μL + 200 μL) with Autonorm containing EDTA, the remaining samples were diluted eleven-fold (20 μL + 200 μL). Corresponding dilution factors were embedded in the batch template in the analysis software.

Sample preparation was performed in 2 mL deep well plates with round wells (Thermo Scientific, cat no 278752). Twenty microliters of calibrator, QC or patient sample was pipetted into the wells. Then, 500 μL cold precipitation solution with IS was added to each well, and the plate was heat-sealed with aluminum foil (Porvair # 229572) using a Porvair Minisessel II heat sealer (Porvair Sciences, King’s Lynn, UK). The plates were centrifuged for six minutes (1400 rpm, 3 mm orbit; High-Speed Multi Plate Shaker, BioSan), and centrifuged for 10 min (2000 g, 4 °C; Rotanta, Hettich). Subsequently, the plates were transferred to the LC–MS/MS autosampler thermostated at 10 °C.

3. LC–MS/MS instrumentation and analytical methods

Mass spectrometric conditions for the analytes (busulfan and PEG 400) were optimized manually using syringe pump side-infusion of analyte solutions into a mobile phase stream of relevant composition and flow rate. Sample analysis was performed on a Transcend II LX-2 UHPLC-system including two-channel LC with separate injection ports and exact length tubing (Viper Finger-tight Fittings, Thermo). The injections were alternately directed to the mass spectrometer, thereby allowing overlapping chromatographic runs and reduced batch analysis time. Mass spectrometric detection was performed on a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific). The inlet system was controlled by Aria version 2.1, and the mass spectrometer was controlled using Tune Application version 1.1. Our laboratory has two identical systems of this LC–MS/MS instrumentation. The busulfan assay was validated on both systems, and runs seamlessly on the two systems in the routine. Chromatographic separation was performed at room temperature (23–25 °C) on Raptor Biphenyl columns (30 x 1D 3.0 mm, particle size 5 μm; Restek). The columns were protected by a 0.5 μm in-line filter placed between the injectors and the columns. No guard columns were used. Mobile phases were (A) water and (B) methanol, both with 0.10 % v/v formic acid and 2.0 mmol/L ammonium acetate. The analytes were stacked on the column at 30 % mobile phase B, and eluted by a step gradient to 85 % mobile phase B. Chromatography details are specified in Table 1. Evaporation and ionization was performed using electrospray in positive mode with a spray voltage of 3 400 V. Nitrogen gas flow: Sheath gas 60 AU, auxiliary gas 8 AU and sweep gas 4 AU. Vaporizer and ion transfer tube temperatures were 470 °C and 270 °C, respectively. Settings for selective reaction monitoring are listed in Table 2. The data were acquired and processed using Tracefinder Software version 3.2. The peak area ratio between analyte and IS was used as signal response. A linear calibration model with orio excluded and 1/x^2 weighting was applied. Two microliters supernatant from the upper part of the well was injected. As the LC–MS/MS systems were set up with 25 μL sample loops for the analysis of immunosuppressants, a front volume of 20 μL was used to avoid carry over. Between injections, the needle and injection port were washed with acetoni-trile:methanol:isopropanol 50:25:25 and water:acetonitrile 95:5. A detailed description of the injection program may be found in supplementary file-1.

PEG 400 and busulfan may be quantified simultaneously in the same run. The mass spectrometric conditions for PEG 400 was based on the work of Vijaya Bhaskar et al [18], with some modifications: Quantification was performed using the precursor mass 344.3 (ammonium adduct of the oligomer mass 326) producing the fragment 89.2, and using busulfan-d8 as IS. Qualitatively, the whole PEG 400 envelope was depicted using precursor ion scans to the fragment 89.2.

Phospholipids were qualitatively assessed both by mass transitions, specific or general (184.1 > 184.1), and by precursor ion scans
to the fragment 184.1, methodologically based on the work by Xia and Jemal [19].

As a non-accredited backup-assay, the busulfan method was also cross-validated on two identical Xevo TQ-S micro LC–MS/MS systems from Waters. The inlets consisted of Acquity UPLC I-class systems (binary high pressure mixing). The conditions of chromatography on the Waters systems were a direct copy of the conditions on the Thermo systems with two exceptions: 1) Composition of the strong-wash solution, and 2): As these systems were less tolerant to injecting samples containing high levels (67 %) of methanol, the samples were post preparation diluted, and the samples were injected as a partial loop injection of 5 μL. Post prep dilution was performed by pipetting 20 μL of each well from a plate prepared as described above into wells containing 500 μL of cold mobile phase A on a new 96 deep well plate. The plate was heat sealed and shaken as described above, and placed in the autosampler without a prior centrifugation.

Details of analytical conditions on the Waters systems are given in supplementary file-1. As these Waters systems do not have the two-channel chromatography option, the effective run-time per batch was twice as long.

4. Method validation

Measurements of PEG 400 in plasma were performed semi-quantitatively after a simplified one-day validation procedure.

The busulfan assay was validated with respect to limits of quantification, selectivity, carry-over, presence of ghost peaks, calibration curve, accuracy and precision, dilution integrity, matrix effects and stability in accordance with the guidelines on bioanalytical method validation provided by the European Medicines Agency and the US Food and Drug Administration [7,20]. All validation experiments, except for experiments related to stability, were performed on both of the Thermo LC–MS/MS systems.

Analytical selectivity was assessed using ten anonymized samples from patients conditioned for HSCT without busulfan. Selectivity was also investigated by analysis of a commercial QC containing 22 therapeutic drugs (amikacin, caffeine, carbamazepine, chloramphenicol, clonazepam, cyclosporine, desipramine, diazepam, doxigen, disopyramide, ethosuximide, flecainide, gentamicin, haloperidol, imipramine, lidocaine, lithium, methotrexate, norfipryline, paracetamol, phenobarbitone, phenytoin, primidone, procainamide, quinidine, salicylate, theophylline, tobramycin, valproic acid, vancomycin; Seronorm Pharmaca L-2, Sero). The IS selectivity was additionally investigated by analyzing 8 samples (collected during a complete dose interval) from a patient receiving busulfan orally using a precipitation reagent without IS added.

We assessed carry-over between subsequent injections by analysis of blanks after QCs spiked with analyte concentrations at the upper limit of quantification (ULOQ) and IS at regular concentration. Potential analyte and IS responses in the blanks were calculated relative to the responses in QCs at the LLOQ added regular IS concentration.

The presence of potential late-eluting peaks after the analysis of patient samples was examined by sequential analysis of 47 wells containing mobile phase (30 %B) following the injection of a busulfan sample from a male patient. The same setup was also performed with a sample from a female patient. The presence of potential late-eluting peaks after the analysis of calibrators and controls was examined by the sequential analysis of 36 wells of mobile phase (30 %B) following the analysis of a full set of calibrators and quality controls.

Three separately prepared calibrator curves from the same lot of calibrators were used for selection of calibration model. Calibration curves were examined with respect to the accuracy of back-calculated concentrations vs. nominal concentrations at each level. Calibrator curves from two additional lots of calibrators were used for verification of the calibration model.

Within- and between-series accuracy and precision were determined with spiked QCs at four levels. Between-series (apparent) accuracy and precision were also determined with repeated analysis of our in-house long term QC (patient pool) in series performed during and after the time period of validation. Signal to noise was calculated at the LLOQ. The coefficient of variation (CV) was used as measure of precision.

Dilution integrity was assessed at two dilution levels: by 2-fold and 11-fold dilution of the high QC (4000 ng/mL) in Autonorm containing EDTA. As recommended in the EMA guidelines in cases where excipients are likely to be responsible for matrix effects, dilution integrity of patient samples was further assessed by serial dilution in triplicate of a patient sample collected at the end of infusion, containing 4052 ng/mL of busulfan and 659 mg/L of PEG 400.

Matrix effects were assessed using six anonymized patient samples from transplanted patients not receiving busulfan. The post-preparation addition method was used; with analytes at threefold LLOQ and at ULOQ, and IS at regular concentrations. The matrix factor (MF) was calculated versus non-matrix (mobile phase, 30 % B), and the IS-normalized MF was calculated as the ratio between the analyte MF and the IS MF. The complete matrix effect setup was repeated with patient samples spiked with PEG 400 to a level of 9.1 mg/L and 45.5 mg/L (equivalent to a PEG 400 level of 100 mg/L and 500 mg/L respectively, in patient samples diluted 11-fold before preparation).

Stability of the precipitation reagent stored in a refrigerator (5–8 °C) was investigated at 24 h, at 4 and 7 days, and at 3, 4, 7, 8 and 12 weeks. Acceptance criterion was a busulfan-d8-response (chromatographic peak area) no lower than median 70 % of the busulfan-d8-response from a freshly prepared bottle. The chromatograms were also examined for any rising signal of the analyte (busulfan-d0) as a function of storage time.

Stability of busulfan in the high, medium and low QC stored in a refrigerator was investigated for 24 h, and 2, 3, 4, 5 and 6 days. Stability of busulfan in patient samples stored in a refrigerator was investigated after 2.5 h, 24 h and 3 days using samples collected during two oral dose intervals, a total of 15 patient samples. Stability of busulfan in patient samples at room temperature (23–24 °C) was investigated for 2.5 h and 24 h using samples from two oral dose intervals, a total of 15 patient samples.

Long-term stability of busulfan calibrators, QCs and patient pool (the in-house long term QC) stored at -70 °C was investigated for up to 27 months. The stability of busulfan patient samples stored at -70 °C was investigated for up to 12 months using samples from three complete IV dose intervals, a total of 22 patient samples.

Post-preparative stability of busulfan patient samples in a refrigerator for up to 24 h was investigated. Patient samples from two complete oral dose intervals were included, a total of 15 patient samples. Stability was investigated both using internal calibration (quantification performed against calibrators on the same plate) and by external calibration (quantification performed against calibration performed at time zero).

For comparison with our previous in-house HPLC-UV-method (based on [21]), a total of 200 patient samples from 27 patient series and 84 parallels of the in-house long term quality control (Lot #9, target 776 ng/mL) were analyzed. The comparison was performed within the quantitation limits for the HPLC-UV method, and using leftover patient samples stored at -70 °C until the day of analysis by LC–MS/MS.

Cross-validation between the Thermo-instruments and our two Waters systems was performed by analyzing calibrators, controls
and a total of 9 patient series (67 patient samples) on three separate days. The plates were first analyzed on the Thermo-systems, thereafter split to two separate plates, and analyzed on both Waters systems at the same day. Instrument-related differences in both single sample results and Css-values was calculated, and also carry-over, accuracy and precision on the Waters instruments.

5. Results and discussion

Unless stated otherwise, the results are presented as mean ± SD.

5.1. Chromatographic and mass spectrometric conditions

The low logP value of busulfan (approximately -0.5) indicates that chromatographic retention of this compound on traditional reversed phase columns is challenging. Busulfan tends to elute with a low retention factor (k-value) and with low amounts of organic modifier in the mobile phase. A low organic content in the mobile phase has a negative impact both on compound stacking on the column and on the effectiveness of the electrospray ionization. Elution of the analyte at low k-values may also contribute to contamination of the ion source from matrix components. Therefore, a number of different stationary phases were tested for their retention of busulfan. Results from the testing of chromatography columns are presented in Table 3. All of the tested alkane type columns produced limited retention of busulfan, whereas the phenyl or biphenyl types showed superior retention properties, facilitating the elution of busulfan at high methanol concentrations. Attempts at developing methods based on isocratic or shallow gradient elution were abandoned due to chromatographic separation between busulfan and the deuterated IS, resulting in poor accuracy and precision for patient samples containing high levels of PEG 400. This problematic deuterium isotope effect is a known issue, and has previously been described by others [22]. The analyte vs. IS separation problem was minimized by using the combination of a short column packed with large particles (the 3.0 mm ID x 30 mm Raptor biphenyl column from Restek, with 5 µm particles) and a high step-gradient for elution of analytes. A short column with large particles had additional benefits for this application as it reduced the run-time by allowing high flow during loading, washing and re-equilibration without producing excessive back-pressure (Fig. 1a). Low temperature (no column heating) allowed for higher methanol content in the mobile phase during stacking of the compounds on the column. Elution of the compounds of interest was performed at low flow, facilitating an effective evaporation and ionization in the ion source. Eluting by a step gradient from 30 to 85 % methanol resulted in a near complete co-elution of the analyte and deuterated IS (Fig. 1c) with a k-value of 5 (calculated based on volume, not time). This principle for loading, washing and re-equilibration has proven robust, and has also been adapted to the analysis of atorvastatin using the same pair of columns [23].

The chromatographic run-time per injection was 2 min and 8 s, allowing an effective run-time of 1 min and 10 s per sample in a batch when using two alternating LC-channels. The step height of the gradient was limited to 85 % methanol to prevent phospholipids and other lipophilic compounds entering the mass spectrometer. All major phospholipids eluted between 1.4 and 1.7 min, i.e. eluted to waste in the washout between injections (data not shown). As the PEG 400 envelope covers a wide range of polarities, it proved difficult to completely separate busulfan from PEG 400. Busulfan eluted in the same timeframe as the lower masses of PEG 400, partly co-eluting with the oligomer producing a precursor at mass 344 (ammonium adduct of the oligomer mass 326). A precursor ion scan to fragment 89.2 depicted the elution profile of the whole PEG 400 envelope, and also the characteristic 44 amu spacing between the PEG oligomers (Fig. 2). To reduce the ion suppression and the potential ion source contamination from PEG 400, the samples from patients receiving IV busulfan were pre-preparation diluted with Autonorm.

Conditions for the mass transitions are presented in Table 2. Fragmentation of the precursor at m/z 264.1 produced a major fragment at m/z 151.1 (272.1 > 159.1 for busulfan-d8). No qualifier ions were used in this method, the dwell time for busulfan and the IS was set to obtain 20–25 data points across the chromatographic peaks. PEG 400 (344.3 > 89.2), producing more than 2E5 counts per second (CPS) at LLOQ, was granted 5 ms of dwell time. The combination of
Table 3
Chromatography columns tested for the retention of busulfan.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Column</th>
<th>Particles</th>
<th>Mobile phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Thermo FS</td>
<td>Accucore C8</td>
<td>2.6 μm solid core</td>
<td>10</td>
</tr>
<tr>
<td>2) *</td>
<td>Accucore Polar Premium</td>
<td>“</td>
<td>10</td>
</tr>
<tr>
<td>3) *</td>
<td>Accucore AQ</td>
<td>“</td>
<td>10</td>
</tr>
<tr>
<td>4) *</td>
<td>Accucore Phenyl-X</td>
<td>“</td>
<td>10</td>
</tr>
<tr>
<td>5) *</td>
<td>Accucore PFP</td>
<td>“</td>
<td>12</td>
</tr>
<tr>
<td>6) ACE LTD</td>
<td>ACE 3 Phenyl</td>
<td>3.0 μm fully porous</td>
<td>25</td>
</tr>
<tr>
<td>7) Restek</td>
<td>Raptor Biphenyl</td>
<td>2.7 μm solid core</td>
<td>30</td>
</tr>
<tr>
<td>8) Restek</td>
<td>Raptor Biphenyl</td>
<td>5.0 μm solid core</td>
<td>42</td>
</tr>
</tbody>
</table>

Chromatographic conditions used: Methanol content (% mobile phase B) adjusted to achieve a retention time of busulfan of 1.0 ± 0.1 min (retention factor of 4) under isocratic conditions.
Columns 1 to 7: 2.1 mm ID x 50 mm. Flow 0.5 mL/min at 60 °C.
Column 8: 3.0 mm ID x 30 mm. Flow 0.5 mL/min at room temperature (23–25 °C).

Fig. 2. PEG–400 in patient samples.

a. Chromatograms from a patient sample taken 5 min after end of infusion. Showing the busulfan IS, and the whole PEG–400 envelope, depicted by a precursor ion scan from mass 130 to mass 680 measuring the fragment B9.2. The PEG–400 signal was attenuated x100 to fit on scale.
b. The masses produced from 0.9 to 1.3 min by the precursor ion scan in Fig. 2a, showing the characteristic 44 amu spacing between the PEG oligomers.

these mass spectrometric conditions and the high methanol content in the mobile phase allowed for the injection of a low sample volume (equivalent to 77 mL of plasma) while still producing a signal to noise for busulfan (as calculated by the Tracefinder software) exceeding 300 at the LLOQ (Fig. 1b).

5.2. Sample preparation

As the busulfan assay were to run on LC–MS/MS systems routinely analyzing 200 samples per day for immunosuppressants, the sample preparation was designed to fit the same format as our immunosuppressants-assay [24]. The precipitation reagent was also the same in the two assays, except for different internal standards. Samples from patients receiving IV busulfan were diluted to decrease the content of PEG 400 (several hundred mg/L, Fig. 3) in the first samples after the end of infusion. Pre-preparation dilution was preferred over post-preparation dilution as the former produced more consistent IS responses in the pre-validation tests (data not shown). In our experience, the IS response is a valuable diagnostic parameter in a routine assay. Dilution and pipetting onto the 96 well plate was performed following a predefined, colour-coded plate map (Shown in supplementary file-2). The dilution scheme was customized to fit samples from patients administered busulfan intravenously as a 3 h infusion aiming for a Css of 750–900 ng/mL. For an assay requiring a fast turnaround time, sample preparation based on a single step protein precipitation on 96 well plates, and a direct injection of the supernatant was the obvious method of choice, avoiding time-consuming solvent evaporation and reconstitution. During the first year of routine use (59 analysis days), the median turnaround time from the start of sample preparation until the analytical results were signed for and released from the instrument was 1 h 56 min for analysis containing one patient series, and 2 h 17 min for analysis containing two patient series (9 samples per patient). Danso et al. presented an assay with extremely fast chromatographic runtime, and a total turnaround time of less than one hour for a complete set of samples [9]. This setup however requires equipment not commonly found in routine labs, and also the evaporation of 3 mL of n-butyl chloride per sample followed by reconstitution before injection.

5.3. Quantification of PEG 400 in patient samples

Several studies on the potential ion suppression effects from drug formulation excipients have been published [25,26], but to our knowledge this issue has not been discussed in relation to TDM of IV-busulfan. As a tool in the development of the busulfan assay, we validated the quantification of PEG 400 in the patients receiving IV-busulfan. Quantification of PEG 400 and busulfan was done in the same run, using busulfan-d8 as internal standard for both analytes. The quantification of PEG 400 in plasma was validated for the LLOQ, carry-over, within day accuracy and precision. The validation was performed on both Thermo LC–MS/MS systems and the results were considered satisfactory for performing semi-quantitative measurements of PEG 400 in patient samples (Data shown in supplementary file-3).

Shortly after end of infusion, the PEG–400 concentration in plasma was 400–800 mg/L (Fig. 3). The elimination of PEG 400 paralleled that of busulfan with a half-life of 1.6–3.0 h, in line with the findings by Shafer [27]. This parallel elimination could entail a special risk as the ion suppression effect of PEG 400 is likely to be concentration-dependent and it can thus exert a decreasing influence on the signal response along the time curve. If not adequately corrected by the IS, such a systematically varying matrix effect would lead to incorrect calculations of pharmacokinetic parameters [25,26]. Larger [25] created a decisional tree that may be a
5.4. Selectivity

No analyte or IS interferences were observed in busulfan-free samples from transplant patients (n = 10) and neither in the commercial QC with therapeutic drugs. No interference from busulfan or metabolites was observed with respect to the IS signal in the 8 patient samples prepared and analyzed without IS.

5.5. Carry-over

The carry-over was unacceptably high during the early stages of methods development, probably as a result of the combination of a wide-ranging calibration curve and the injection of a small sample volume into large sample loops. The carry-over was minimized by using a front volume of 20 μL to wash the sample path before loading 2 μL of sample into the loop. A detailed description of the carry-over analysis is available in the Supplemental Information.

Fig. 3. Elimination of busulfan and PEG-400 in patients. Patient 1, 2, 3, 4 and 6 were administered busulfan intravenously as a 3 h infusion aiming for a Css of 900 ng/mL. Patient 5 had a target Css of 750 ng/mL. Busulfan and PEG-400 were quantified simultaneously in the same run.
of the injection program may be found in supplementary file-1. Using these settings, the carry-over between subsequent injections was equal to zero (undetectable) for busulfan and busulfan-d8 (n = 6).

5.6. Presence of ghost peaks

No late-eluting peaks were detected following the injection of patient samples nor after the calibrators and quality controls.

5.7. Calibration curves and linearity

Using seven non-zero calibrators including LLOQ and ULOQ, the simplest calibration model that adequately described the relationship between the instrument response (peak area ratio BUS/IS) and the busulfan concentration was a linear calibration model, weighted 1/x². Origo was not included in the model. The calibration model proved robust over several lots of calibrators (Table 4). The accuracies of all individual back-calculated concentrations were within 94.8 %–107 %.

5.8. Accuracy and precision

The mean accuracy of QC samples ranged from 101 to 108 %, and CVs were ≤ 3.4 %, verified on two LC–MS systems (details in Tables 5a and 5b). During the period of validation of the busulfan assay, a total of 84 determinations over 24 analysis days of the in-house long term quality control (Lot #9, target 776 ng/mL) produced a mean apparent accuracy of 98.7 % and a CV of 3.5 %. During a total of 64 analysis days over a period of 18 months of routine use, and with seven different operators, the in-house long term quality control (Lot #10, target 955 ng/mL) produced a mean apparent accuracy of 101 % and a CV of 3.7 % (n = 64). Analysis of the external quality control series from the Canseach laboratory produced a blank result for the null sample, and a median accuracy of 95.3 % (range 92.9 %–99.6 %) for the spiked samples, with deviations evenly distributed over the concentration range 125 ng/mL to 4000 ng/mL. The 2019 year report from SKML showed a trueness of +8.1 % and a precision of 1.4 %.

External quality control schemes are important tools in documenting and maintaining assay quality. We found no quantifiable amounts of PEG 400 in any of the samples from the external quality control series from the Canseach laboratory [17] or the samples received from SKML in 2019. These observations suggest that none of the external controls originated from patients receiving IV-busulfan. The clinical representativeness of the external control material would be improved if it also included patient samples collected during the first hours after an intravenous busulfan infusion. Potential matrix effect challenges that will not be detected by comparison of spiked sample results could then have been identified by participation in the proficiency testing program.

5.9. Limits of quantification

The upper limit of quantification was set at 5000 ng/mL, and LLOQ was set at 25 ng/mL as this range was considered adequate by users of our busulfan analytical service. The results for accuracy, precision, selectivity and carry-over all allowed for an LLOQ at 25 ng/mL. Signal-to-noise ratios at LLOQ were ≥ 300 (n = 12 in two series), suggesting that an LLOQ lower than this may be possible (Fig. 1b). This option was not pursued further in this method validation.

5.10. Dilution integrity

The high level QC analyzed undiluted was mean 4049 ng/mL (n = 8, CV 2.9 %). The same control diluted twofold before analysis was mean 4017 ng/mL (n = 8, CV 2.1 %) and diluted eleven-fold the measured concentration was mean 3836 ng/mL (n = 8, CV 1.9 %). A mean recovery of 95.9 % for the sample diluted eleven-fold was well within the recovery limit 85–115 % as recommended by EMA.

Matrix effects from drug formulation excipients may potentially compromise the dilution integrity of patient samples. As pointed out by Larger et al. [25] and in the EMA guidelines [7], spiking experiments may not fully reveal the potential for ion suppression caused by formulation agents due to metabolism or in vivo degradation of the formulation excipients. As about 25 % of an intravenous dose of PEG 400 is metabolized [27], dilution experiments with actual patient samples collected shortly after end of infusion should be performed to reveal dilution or suppression issues with busulfan patients samples. Fig. 4 shows strong ion suppression, calculated as relative IS area (IS area of sample x 100/mean IS area of calibrators). In the undiluted sample the relative IS response was mean 34 ± 6 %, rising with increasing dilution factor to 93 ± 4 % at a dilution factor 11. The strong suppression effect did not, however, have any negative impact on the quantification of busulfan, indicating that the ion suppression on the busulfan signal is fully corrected by the co-eluting IS.

5.11. Matrix effects

Significant ion enhancement (matrix factor 1.2 ± 0.2) was demonstrated for busulfan at high analyte concentration. This was however corrected by the IS. The IS-normalized matrix factor was 1.00 ± 0.04 at low analyte concentration and 0.98 ± 0.06 at high analyte concentration. The addition of PEG 400 to a level of 9.1 mg/L and 45.5 mg/L produced no matrix effects, but attenuated the ion enhancement at high analyte concentration to a matrix factor of 1.00 ± 0.02. The IS-normalized matrix factor in the samples spiked with 45.5 mg/L of PEG 400 (equivalent to a PEG 400 level of 500 mg/L in a patient sample diluted 11-fold before preparation) was 0.96 ± 0.07 (CV 7.5 %) at low analyte concentration and 0.94 ± 0.08 (CV 8.9 %) at high analyte concentration. These results indicate that the

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Table 4: Calibration curves and linearity.

<table>
<thead>
<tr>
<th>Nominal [ng/mL]</th>
<th>Run 1 Cal lot 1</th>
<th>Run 2 Cal lot 1</th>
<th>Run 3 Cal lot 1</th>
<th>Accuracy (n = 3) Cal lot 1</th>
<th>Run 1 Cal lot 2</th>
<th>Run 1 Cal lot 3</th>
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<tr>
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<td>2075</td>
<td>102.4</td>
<td>2011</td>
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<td>5028</td>
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<td>1.000</td>
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Table 5a
Accuracy and precision on LC–MS/MS System 1.

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<th>Nominal conc. (ng/mL)</th>
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<td></td>
<td>Mean accuracy (%)</td>
<td>CV (%)</td>
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Table 5b
Accuracy and precision on LC–MS/MS System 2.

<table>
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<th>Nominal conc. (ng/mL)</th>
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<th>Between series</th>
</tr>
</thead>
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<td>Mean accuracy (%)</td>
<td>CV (%)</td>
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<tr>
<td>4000</td>
<td>101</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Fig. 4. Dilution integrity.
A patient sample collected at the end of infusion, containing 4052 ng/mL of busulfan and 659 ng/mL of PEG 400, was serially serial diluted in autornon before sample preparation. The experiment was performed in triplicate on two different LC–MS/MS instruments. Data are presented as mean ± 1SD, n = 6.

The stability of busulfan in plasma is temperature dependent [29]. Our lab analyzes 40–60 busulfan patient series per year as a service for the clinical routine. To facilitate routine use of this assay, we prefer to prepare batches of ready-to-use series of calibrators and controls in sufficient quantities for at least one year. To maximize long term stability, these batches are stored at ≤ −70 °C, hence we did not validate stability in ordinary freezers (−18 to −20 °C). Storing or handling busulfan calibrators and controls at room temperature should be minimized, this storage temperature was therefore validated only to a limited extent.

Busulfan QCs stored in a refrigerator for up to 6 days showed a loss of 2.1 ± 1.8 % at 48 h, 4.3 ± 1.9 % at 3 days and 9.5 ± 2.8 % at 6 days (144 h) (n = 3 at each concentration level). Although the stability was documented to be 6 days in a refrigerator according to the formal acceptance limit (loss of less than 15 %), in the routine laboratory the recommended shelf life in a refrigerator was set to 48 h, or 2 consecutive analysis days.

Busulfan patient samples in plasma stored in a refrigerator showed a loss of 1.3 ± 5.4 % at 2.5 h, 1.9 ± 5.1 % at 24 h and 8.3 ± 4.5 % at 3 days (72 h) (n = 15 at each time point). In the routine analysis setting, the recommended shelf life for patient plasma samples stored in a refrigerator was set to 24 h. Storage for up to 72 h was allowed, then considering an expected 8 % loss of busulfan.

Busulfan patient samples in plasma stored at room temperature (23–24 °C) showed a loss of 2.5 ± 5.0 % at 2.5 h and 23.4 ± 3.5 % at 24 h (n = 15 at each time point). In the routine analysis, the maximum shelf life for patient plasma samples stored at room temperature was set to 2.5 h, with a recommendation to minimize any storage of busulfan patient samples in plasma at room temperature.

Busulfan QCs at three levels stored in an ultrafreezer showed a loss of 1.5 ± 2.1 % after 12 months, 1.4 ± 3.4 % after 15 months, 0.1 ± 4.9 % after 20 months and 0.2 ± 6.7 % after 27 months (n = 18 at each time point). The formal maximum shelf life for quality controls stored in an ultrafreezer was set to two years (24 months).

Busulfan calibrators stored in an ultrafreezer showed a loss of 3.7 ± 2.5 % after 12 months, 1.6 ± 3.8 % after 15 months, 3.1 ± 3.9 % after 20 months and 5.5 ± 4.9 % after 27 months (n = 21 at each time point). The recommended shelf life for calibrators stored in an ultrafreezer was set to one year (12 months). The formal maximum shelf life for calibrators stored in an ultrafreezer was set to two years (24 months).

The QCs were more stable during long term storage in an ultrafreezer than the calibrators, even though they were prepared simultaneously and in the same matrix. The only difference being the fill grade of the tubes, 60 % for the calibrators (300 μL aliquots in 0.5 mL tubes) vs 100 % for the QCs (500 μL aliquots in 0.5 mL tubes).

The in-house long term QC (patient pool, Lot #9, target 776) stored at ≤ −70 °C showed a loss of 2.1 ± 1.4 % (n = 12) at 26 months. Lot #10, target 955 ng/mL showed a loss of 0.7 ± 1.6 % at 1 month, 0.0 ± 0.7 % at 10 months and an increase of 1.0 ± 2.1 % at 17 months (n = 6 at each timepoint). The formal maximum shelf life for the in-
house long term QC stored in an ultrafreezer was set to two years (24 months).

Busulfan patient samples stored at ≤ −70 °C showed a loss of 1.8 ± 4.5 % after 12 months (range −11.2 to +5.0 %, n = 22). The shelf life for patient samples stored in an ultrafreezer was set to one year (12 months).

In the present study, patient samples (single samples or pools) were more stable during long term storage in ultrafreezers than what have been reported in other studies. Balasubramanian [30] found a loss of 7 % after one year and 8 % after two years. Choong [17] found a loss of 14 % after two years and 15 % after four years. The reasons for these discrepancies remain unknown, but referring to our findings regarding the stability-differences between calibrators and controls, it is tempting to speculate that fill grade in the tubes used for storage may be a factor.

Prepared patient samples of busulfan were stable in a refrigerator for up to 24 h. Reanalysis using internal calibration (quantification performed against re.injected calibrators on the same plate) produced an accuracy of 98.3 ± 4.9 % (n = 15). Reanalysis using external calibration (quantification performed against calibration performed at time zero) produced an accuracy of 97.5 ± 4.7 % (n = 15). Given that the plate with prepared busulfan patient samples was stored cold, samples on the plate may be re injected and quantified for up to 24 h without running a new calibration. Also the plate may, if necessary, be stored under cool conditions for up to 24 h awaiting analysis.

Repeated freezing and thawing of patient samples was not validated by us. Nadella [15] found busulfan to be stable for at least 5 freeze/thaw cycles, Danso [9] found busulfan to be stable for at least 6 freeze/thaw cycles, and French [11] found busulfan to be stable for at least 8 freeze/thaw cycles.

5.13. Methods comparison UHPLC-MSMS vs HPLC-UV

The mean difference between the analytical methods (UHPLC-MSMS vs HPLC-UV) for 200 patient samples was +0.5 % with a 95 % confidence interval from −2.5 % to +3.4 %. The HPLC-UV in-house long term QC analyzed by LC-MSMS produced an inaccuracy of −1.3 % with a 95 % confidence interval from −2.1 % to −0.6 % (n = 84).

5.14. Cross-validation on waters acquity UPLC i-class/Xevo TQ-S systems

Busulfan levels in the patient samples were mean 4.5 % higher (range -12.8 % to +15.1 %, n = 67) when the same samples were analyzed on the Waters systems. The Css-values calculated based on the results from the Waters instruments were also slightly higher, mean difference +3.9 % (range +2.5 % to +5.6 %, n = 9 for each instrument). These differences were however considered to be of minor importance in a busulfan TDM setting. Carry-over was undetectable, accuracy ranged from 101 % to 105 % for the QC and all CVs were <5.0 %. Based on these results, busulfan analysis on the two Waters systems was approved as a non-accredited backup-assay.

5.15. Clinical application

The present assay has been applied in clinical routine for the personalization of busulfan dosing in a total of 113 pediatric and adult patients who were administered IV busulfan for pre-conditioning prior to HSCT. According to current protocols, busulfan is given for four days as a 2–3 h infusion. Individual pharmacokinetic parameters are calculated based on the analysis of nine samples collected during the first dose interval. The reliability and speed of the present assay allowed results including dose adjustment recommendations to be reported well in advance of the next dose. When large dose adjustments were made (>20 %), attainment of target concentrations could be confirmed following subsequent sampling and analysis. The robustness of the method allowed availability of such service seven days per week.

6. Conclusions

We have developed a fast and simple method for quantifying busulfan across a wide concentration range requiring only 20 µL of blood plasma. The method has been validated in accordance with the EMA and FDA guidelines, to our knowledge the first busulfan assay validation that takes into account the potential ion suppression effects from drug formulation excipients. The assay performs well across instrument platforms, it has been implemented in routine practice with accreditation according to the ISO 15189 standard, and performs well in external quality control assessments.

Author statement

Anders M Andersen: Conceptualization, Investigation, Writing - original draft, Methodology. Stein Bergan: Conceptualization, Investigation, Writing - original draft, Methodology, Resources, Supervision. Tobias Gedde-Dahl: Conceptualization, Writing - review & editing, Resources. Jochen Buechner: Conceptualization, Writing - review & editing, Resources. Nils Tore Vetle: Conceptualization, Investigation, Writing - original draft, Methodology, Supervision.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114216.

References


