Sensitive and Rapid Quantification of Busulfan in Small Plasma Volumes by Liquid Chromatography–Electrospray Mass Spectrometry

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Background: High-dose busulfan is widely used in conditioning regimens before hematopoietic stem cell transplantation in both adults and children. Large interindividual variability in pharmacokinetics after oral administration has been reported; therefore, therapeutic drug monitoring of busulfan may decrease the incidence of drug-related toxicity (for example, hepatic venoocclusive disease) and may also improve therapeutic efficacy.

Methods: Busulfan concentrations were quantified using 200 μL of plasma and liquid–liquid extraction with diethyl ether after the addition of [2H8]busulfan as the internal standard. Separation and detection of busulfan and [2H8]busulfan were achieved with a LUNA C8 column (5 μm; 150 × 2 mm i.d.) at 30 °C, a HP 1100 liquid chromatography system, and a HP 1100 single-quadrupole mass spectrometer. Busulfan and [2H8]busulfan were detected as ammonium adducts in selected-ion monitoring mode at m/z 264.2 and 272.2, respectively.

Results: The calibration curve was linear at 5–2000 μg/L busulfan. Intra- and interassay imprecision (CV) and bias were both <11%. The limits of detection and quantification were 2 and 5 μg/L, respectively. Extraction recovery of busulfan was >87%. Analysis of pharmacokinetics in four patients receiving high-dose busulfan indicated that minimum busulfan concentrations before the next dose were 405–603 μg/L, with no interference observed.

Conclusions: The new rapid and sensitive liquid chromatographic–mass spectrometric assay is an appropriate method for quantification of busulfan in human plasma, making therapeutic drug monitoring of busulfan faster and easier in clinical practice.

High-dose busulfan is widely used as a conditioning treatment before hematopoietic stem cell transplantation (HSCT) in both adults and children (1). Because of highly variable bioavailability after oral administration, plasma concentrations of busulfan display high inter- and intra-individual variability (2). Consequently, using a standard dosing regimen of busulfan of 4 mg·kg⁻¹·day, patients with lower plasma concentrations and lower systemic exposure have been reported to have an increased risk of engraftment failure and late relapse after bone marrow transplantation or HSCT (3, 4). In contrast, high systemic exposure of busulfan has been correlated with an increased risk of drug-related toxicity. For example, the incidence of hepatic venoocclusive disease, a hepatic lesion involving obstruction of small intrahepatic venules and subsequent damage to the surrounding centrlobular hepatocytes, was observed in up to 20–40% of children and adults who underwent bone marrow transplantation (5, 6). Therefore, therapeutic drug monitoring (TDM) has been considered of benefit for individual optimization of busulfan therapy (7–9).

Different HPLC and gas chromatographic methods for the determination of busulfan in plasma and other biologic fluids have been described previously (10–17), but these have several disadvantages. For example, because of the limited absorbance of busulfan over the ultraviolet
wavelength spectrum, derivatization of busulfan and the internal standard before assay is necessary (10–12, 14). In addition, there are some limitations in respect to the sensitivity of previously described methods. For example, HPLC analyses have limits of quantification between 50 and 60 μg/L (11, 12, 14, 15), whereas two gas chromatographic methods (13, 16) have limits of quantification of 20 and 40 μg/L, respectively. Furthermore, with the exception of only two methods (12, 15), 1 mL of plasma is required as a minimum sample volume, which seems to be unsuitable for clinical TDM in small children. However, gas chromatographic analysis is time-consuming because sample preparation takes 3–4 h. We describe here a relatively simple, rapid, and sensitive liquid chromatography–mass spectrometry (LC-MS) assay for quantification of busulfan in human plasma.

**Materials and Methods**

**Materials**

Busulfan was purchased from Sigma. The internal standard, [2H₈]busulfan, was obtained from Euriso-Top. Other materials were obtained from the following sources: JT Baker (acetonitrile), Merck (acetic acid, ammonium acetate, and sodium chloride); Promochem (diethyl ether); and Fluka Chemie AG (dimethyl formamide).

**Solutions**

Stock solutions of busulfan and [2H₈]busulfan were prepared in dimethyl formamide at a concentration of 10 g/L. Working solutions were diluted in acetonitrile to obtain final concentrations of 1 mg/L, 10 μg/L, and 1 μg/L busulfan. A solution of [2H₈]busulfan in acetonitrile (20 mg/L) was used as the internal standard. All stock solutions were stored at −20°C for up to 3 months. Calibrators at concentrations of 5–2000 μg/L were prepared by supplementing busulfan working solutions with 200 μL of busulfan-free plasma. Quality controls were obtained by adding busulfan to 10 mL of blank plasma to obtain final concentrations of 5, 10, 100, 250, and 2000 μg/L. Aliquots were frozen at −20°C and analyzed together with patient samples. Two mobile phases, designated A and B, were as follows: 10 mmol/L ammonium acetate and 10 mL/L acetic acid in water (A) and acetonitrile (B).

**Sample Preparation**

Internal standard (10 μL; 20 mg/L [2H₈]busulfan) and 300 μL of a saturated solution of sodium chloride were added to 200 μL of plasma in a 10-mL glass tube. The samples were extracted with 4 mL of diethyl ether by shaking for 15 min and centrifuging for 5 min at 1200g. The organic layer was evaporated to dryness under a stream of nitrogen at 37°C. The residue was dissolved in 120 μL of a mixture of mobile phase A–mobile phase B (85:15 by volume).

**LC-MS Analysis**

Chromatographic separation of busulfan and [2H₈]busulfan was performed using a LUNA C₈ analytical column (5 μm particle size; 150 × 2 mm i.d.; Phenomenex) at a column temperature of 30°C, and a HP 1100 LC system (Agilent Technologies) with a binary pump. Gradients were programmed as follows (flow rate, 0.5 mL/min): 15% mobile phase B for 2 min, increased to 50% B for 6 min, decreased to 15% B for 1 min, and then reequilibrated with 15% B for 2 min, until the next sample was injected. A column-switching valve was used to switch the liquid flow into the detector 4–8 min after injection.

A HP1100 single-quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospary source was used. Electrospray settings were as follows: spray voltage, 2000V; fragmentor, 60V; gas temperature, 345°C; nebulizer pressure, 50 psi; drying gas, 11 L/min. Positive ionization with selected-ion monitoring mode was used, and the multiplier gain was set on 1. Busulfan and [2H₈]busulfan were detected as ammonium adducts at m/z 264.2 and 272.2, respectively. Peak areas of busulfan and [2H₈]busulfan were analyzed and subsequent calculation of calibration curves and quantification of busulfan was performed with HP Chemstation software (Ver. 98; Agilent Technologies).

**Assay Validation**

Calibration curve samples with final concentrations of 10, 20, 100, 500, 1000, and 2000 μg/L busulfan were prepared as described above. Calibration curves were obtained by plotting the peak area ratio m/z 264.2/272.2 of busulfan and [2H₈]busulfan, respectively, against the initial busulfan concentrations. Intraday accuracy and precision were assessed by assaying five replicates each of 5, 10, 25, and 250 μg/L busulfan quality-control samples on a single assay day. Interday accuracy and precision were determined by analysis of 10, 100, and 2000 μg/L busulfan quality-control samples on 6 different assay days. Calibration curves were obtained by plotting the peak area ratio m/z 264.2/272.2 of busulfan and [2H₈]busulfan, respectively, against the initial busulfan concentrations. The limit of detection was defined as the concentration that gave a signal-to-noise ratio of 3, and the limit of quantification was determined as the lowest concentration with a CV of <20% (n = 5).

Recovery of busulfan was investigated by adding known concentrations (10, 100, and 2000 μg/L) of busulfan to blank plasma samples and extracting the busulfan. Peak area ratios were compared with those obtained by direct injection of the corresponding busulfan concentrations.

Long-term stability (3 months) of busulfan in plasma at −20°C was investigated via repeated analysis of patient samples. In addition, the stability of busulfan in plasma at 4°C and at room temperature for up to 6 days was assessed, as well as the stability of busulfan in extracted samples at room temperature and at −20°C.
Clinical samples
Four patients with different kinds of malignancies [high-grade non-Hodgkin lymphoma (n = 2), Hodgkin disease (n = 1), and plasmocytoma (n = 1)] were treated with Busulfex™ [6 g/L solution of busulfan dissolved in N,N-dimethylacetamide–polyethylene glycol (33:67 by volume); Orphan Medical] in conditioning regimes for HSCT. Three patients were male and one was female (mean age, 37.8 years; age range, 16–51 years; refer to Table 1 for demographic data), and all patients gave written informed consent before the study. Plasma samples were collected before administration of the eighth dose of busulfan, given as an infusion over 2 h and at 0, 0.5, 1, 2, 3, 4, 6, 12, 18, and 26 h after the end of the infusion. Blank human plasma was obtained from healthy volunteers. Samples were stored at −20 °C until further processing.

Data analysis
Stability of busulfan after 3 months storage was analyzed with non-least-squares linear regression (GraphPad PRISM, Ver. 2.0; GraphPad Software Inc.). Pharmacokinetic data were calculated according to the standard noncompartmental model with the TopFit 2.1 program (18).

Results
Electrospray ionization conditions
Different mobile phases were tested to optimize the sensitivity of the electrospray ionization. There was only a minor influence of the organic solvent used (500 mL/L methanol vs 500 mL/L acetonitrile). In the presence of acetic acid, the spectra showed a base peak at m/z 269.2 [M + Na⁺] with additional peaks at m/z 247.2 [M + H⁺], m/z 264.2 [M + NH₄⁺], and m/z 285 [M + K⁺] of variable intensity. When we used 10 mmol/L ammonium acetate–acetonitrile (50:50 by volume) as the solvent, the base peak was [M + NH₄⁺] with other peaks [[M + Na⁺] and [M + K⁺]] of only minor intensity. However, the overall sensitivity was insufficient. Decreasing the pH of the buffer solution by the addition of 10 mL/L acetic acid markedly improved signal height to optimize the sensitivity.

Chromatographic separation
Chromatograms resulting from the extractions of a blank plasma sample, a quality-control sample at the limit of quantification (5 μg/L), and plasma samples from one patient are shown in Fig. 1. Under the LC-MS conditions described, the retention times of busulfan and [²H₈]busulfan were 5.8 and 5.7 min, respectively. Because of the highly selective detection method, there were no interfering peaks present in 10 blank samples investigated in the time window of 4–6.5 min when the analytes were

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**Table 1. Patient demographics.**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age, years</th>
<th>Body weight, kg</th>
<th>Dose</th>
<th>Body surface area, m²</th>
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</thead>
<tbody>
<tr>
<td>171/A</td>
<td>Male</td>
<td>51</td>
<td>73</td>
<td>50</td>
<td>0.7</td>
</tr>
<tr>
<td>171/B</td>
<td>Male</td>
<td>40</td>
<td>100</td>
<td>80</td>
<td>0.8</td>
</tr>
<tr>
<td>171/C</td>
<td>Female</td>
<td>16</td>
<td>52</td>
<td>40</td>
<td>0.8</td>
</tr>
<tr>
<td>171/D</td>
<td>Male</td>
<td>44</td>
<td>76</td>
<td>54</td>
<td>0.7</td>
</tr>
</tbody>
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Fig. 1. Representative chromatograms from extractions of a blank plasma (A); 5 μg/L busulfan and 400 μg/L [²H₈]busulfan (B); and a patient plasma sample (C).
eluted from the column. The gradient program was used to ensure that all contaminants were washed from the column within a run time of 10 min, ensuring a prolonged life of the analytical column. No deterioration of the column was observed in 350 injections of the extracted plasma samples.

ASSAY VALIDATION
Calibration curves were linear over the concentration range used for busulfan, with correlation coefficients of 0.9992–0.9999 (n = 4). The mean (± SD) y-intercept was 0.000975 (±0.00692), and the mean (± SD) slope was 0.00093 (±0.000007) over 4 independent assay days.

The accuracy and precision values are shown in Table 2. The limit of detection and limit of quantification were 2 and 5 µg/L, respectively. Recoveries of 10, 100, and 2000 µg/L busulfan after extraction were >87% (Table 3).

Busulfan is known to react with nucleophilic groups, such as amines, in a substitution reaction. This reaction may occur during storage; therefore, the stability of busulfan in plasma samples under different conditions was investigated. After 3 months of storage at −20 °C, the busulfan concentration differed by a mean of 2.9% (range, −7.7% to 12.6%) from that originally quantified (concentration range, 9–1895 µg/L), indicating long-term stability. Furthermore, because there is a possibility that samples may be left accidentally at room temperature or in a typical refrigerator for some time during acquisition or processing, stability at 4 °C and at room temperature was investigated. The degradation of 105 µg/L busulfan in plasma under these conditions is shown in Fig. 2. Although busulfan is stable for up to 24 h at 4 °C, the busulfan concentration decreased to ~75% within 24 h at room temperature. After 6 days at room temperature, only 20% of the initial concentration of busulfan remained. Extracted samples redissolved in mobile phase were stable for 12 h at room temperature and for up to 1 month at −20 °C.

CLINICAL SAMPLES
Steady-state plasma concentrations (C_{SS}s) of busulfan at and after the administration of the eighth busulfan dose are shown in Fig. 3. The mean minimum busulfan plasma concentration before the eighth dose in the four patients was 497 µg/L (range, 405–603 µg/L). The mean area under the plasma concentration–time curve [(AUC); 0–26 h] for busulfan was 1839 µmol/L·min. Plasma concentrations of busulfan displayed linear pharmacokinetic behavior with a mean elimination half-life of 3.6 h. Complete pharmacokinetic data of all patients are given in Table 4.

Discussion
In the last 10 years, pharmacokinetically guided administration of chemotherapeutic agents has become of increas-

<table>
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<th>Table 2. Intra- and Interday accuracy and precision of busulfan samples.</th>
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<td>Actual concentration, µg/L</td>
</tr>
<tr>
<td>Intraday (n = 5)</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>250</td>
</tr>
<tr>
<td>Interday (n = 10)</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>2000</td>
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<th>Table 3. Recovery of busulfan from extracted plasma samples.</th>
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<tr>
<td>Busulfan added, µg/L</td>
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<tr>
<td>----------------------</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>2000</td>
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*All data are mean ± SD from five determinations.*
ing interest because the assessment of pharmacokinetic parameters such as AUC, drug $C_{\text{ss}}$, and drug exposure time above a certain plasma concentration may lead to less toxicity and higher efficacy compared with administration on the basis of body surface area only. For example, AUC-guided administration has been investigated extensively for carboplatin, leading to a more precise prediction of the effect of carboplatin in terms of dose-limiting toxicity mainly as thrombocytopenia.

Busulfan has been used since the 1950s, and currently it is one of the most frequently used chemotherapeutic agents in high-dose regimes for patients undergoing HSCT (1). The majority of clinical studies investigating pharmacokinetics of busulfan have demonstrated a pharmacodynamic relationship between busulfan $C_{\text{ss}}$ and outcome in patients with busulfan–cyclophosphamide preparative regimens in HSCT, as well as busulfan-related toxicity (for example, venoocclusive disease or intestinal pneumonia) (9). Thus, several investigators have recommended TDM of busulfan to maximize the likelihood of engraftment and reduce busulfan-related toxicity (9, 22).

This report describes a new, highly sensitive and specific LC-MS assay for the quantification of busulfan in small volumes of patient plasma. This method requires only 200 µL of human plasma for accurate determination of busulfan concentrations because of a low limit of quantification (5 µg/L), which is 10-fold lower than the majority of previous reports (11, 12, 14, 15). Furthermore, this volume is more appropriate when attempting TDM of busulfan concentrations in children when conducting AUC-guided administration.

A further advantage of this method is that it does not require a derivatization step in contrast to the majority of methods described previously (10–16, 23). In addition, a simple liquid–liquid extraction was sufficient to produce chromatography free from interference of endogenous compounds in plasma. Although most previous methods have also used liquid–liquid extraction, the present method used diethyl ether as the solvent in comparison with diethyl ether–dichloromethane (10), ethyl-acetate (11–13, 16), toluene (14), or dichloromethane (23). Of all of these solvents, diethyl ether is the quickest to evaporate under $N_2$ at a lower temperature and, compared with chlorinated solvents, has a lower environmental hazard potential. Taken together, the use of diethyl ether for the extraction of busulfan from plasma and the absence of a derivatization step markedly reduce assay time. Zuccaro et al. (17) described a LC-MS method using particle beam evaporation together with electron-impact ionization. In addition to a comparatively low sensitivity with a limit of quantification of 100 µg/L, this kind of LC-MS system is too temperamental for routine laboratory use.

Apart from assay duration, accuracy and precision as well as extraction recovery must also be considered when assessing the quality of any particular method. Intra- and interassay validation data demonstrate that even at the limit of quantification of the present assay, accuracy and precision are retained (see Tables 2 and 3). Recovery of the internal standard, [1H3]busulfan, was not assessed because of the high degree of similarity with busulfan; however, recoveries of busulfan were 87–92% over a concentration range of 10–2000 µg/L. Previous methods have reported recoveries of 90–102% (10, 12–16). Therefore, it can be concluded that the method of extraction in the present assay, although quick and simple, is just as effective as previous methods used. Unless blood samples are left at room temperature or in a typical refrigerator for more than a few hours or up to 1 day, respectively, the described method is well suited for the rapid and accurate TDM of busulfan during conditioning before HSCT. Additionally, the new formulations of intravenous busulfan, such as Busulfe (dissolved in dimethylacetamide) and a liposomal form, require clinical trials that include an evaluation of pharmacokinetic parameters, especially in children. Thus, the present method may also be helpful for clinical research.

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References