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A fast and simple assay for busulfan in serum or plasma by liquid chromatography–tandem mass spectrometry using turbulent flow online extraction technology

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ABSTRACT

Busulfan is used in myeloablative preparation regimens for hematopoietic bone marrow transplantation. Due to its narrow therapeutic range therapeutic drug monitoring of busulfan is recommended. In this study a fast and simple method for measuring busulfan in serum or plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed utilizing turbulent flow online extraction technology. Serum or plasma was mixed with acetonitrile containing d_8-busulfan. After centrifugation the supernatant was injected onto a turbulent flow preparatory column then transferred to a C18 analytical column monitored by a tandem mass spectrometer set at positive electrospray ionization. The analytical cycle time was 4.0 min. The method was linear from 0.15 to 41.90 μmol/L with an accuracy of 87.9–103.0%. Inter- and intra-assay CVs across four concentration levels were 2.1–7.8%. No significant carryover or ion suppression was observed. No interference was observed from commercial control materials containing more than 100 compounds. Comparison with a well established LC–MS/MS method using patient specimens ($n = 45$) showed a mean bias 1.3% with Deming regression of slope 1.02, intercept −0.02 μmol/L, and a linear correlation coefficient 0.9883. The LC–MS/MS method coupled with turbulent flow online sample cleaning technology described here offers reliable busulfan quantitation in serum or plasma with minimum manual sample preparation and was fully validated for clinical use.

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1. Introduction

Busulfan is an alkylating agent commonly used in myeloablative preparative regimens for hematopoietic bone marrow transplantation [1]. Oral dosing presents highly variable pharmacokinetics with a challenging balance between therapeutic and toxic effects [1–4]. Though intravenous dosing provides better survival and more predictable pharmacokinetics [1,5–7] many factors including age and co-administered drugs can affect the pharmacokinetics significantly [1,8,9]. Therefore therapeutic drug monitoring of busulfan is recommended [1,8].

Busulfan has been measured by gas chromatography or high-performance liquid chromatography (HPLC). Due to the low absorption in the ultraviolet/visible range and low volatility, busulfan methodologies except liquid chromatography–mass spectrometry (LC–MS) or LC–tandem mass spectrometry (LC–MS/MS) require derivatization and tedious sample preparation [10–18]. LC–MS or LC–MS/MS methods offer the highest sensitivity and require the smallest sample sizes [1]. However, many of these methods use liquid–liquid extraction or protein precipitation followed by evaporation and reconstitution steps that increase the sample preparation time [19–22]. Online solid-phase extraction has also been reported for LC–MS/MS measurement of busulfan [23,24]. A short LC–MS/MS method with simple sample preparation was reported by Chen et al. with a linear range of 1.096 to 1096 ng/mL (0.004–4.46 μmol/L) [25]. However, the reported peak concentrations may range from 11.0 to 21.5 μmol/L via daily IV administration [6].

Turbulent flow technology is a robust and rapid online purification tool for high efficiency extraction across a large concentration range [26]. Here we report a novel LC–MS/MS method using turbulent flow online extraction technology for sample purification prior to quantification of busulfan in serum or plasma by LC–MS/MS. This method is quick (4 min) and robust with a simple sample preparation step (protein precipitation) and has a linear range of 0.15–41.90 μmol/L.

Abbreviations: HPLC, high performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry.

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2. Materials and methods

2.1. Reagents and solutions

Methanol, acetone, acetonitrile, and isopropanol (Burdick and Jackson High Purity Solvent) were from VWR (West Chester, PA, USA). Type 1 water was from a Millipore Synergy System (Billerica, MA, USA). Busulfan was ≥99% (Sigma–Aldrich, St. Louis, MO, USA). Tetramethylene-d_8 Busulfan (99%-^{2}H) was procured from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Commercial drug free serum was obtained from Bio-Rad (Hercules, CA, USA). A turbo preparatory column, Cyclone-P 0.5 mm × 50 mm, and a C18 column, Hypersil GOLD 3.0 mm × 50 mm, 5 μm, were purchased from Thermo Fisher (Waltham, MA, USA). A solvent mixer (P/N G112-87330) was from Agilent (Santa Clara, CA, USA). The HPLC sampling vials and inserts were from Sun International (Wilmington, NC, USA).

A stock solution for both busulfan [(y) nmol/L = 4.065*(x) ng/mL] in acetone and d_8-busulfan [(y) nmol/L = 3.937*(x) ng/mL] in acetonitrile was made at a concentration of 2032.5 μmol/L and 393.7 μmol/L, respectively. The calibration standard was made at 20.33, 10.16, 5.08, 2.54, 1.27, 0.64, 0.32, and 0.00 μmol/L, and was prepared by serial dilution in commercial drug-free blank serum and stored at −20 °C. A precipitation solution containing d_8-busulfan at 3.94 μmol/L in methanol was stored at 2–8 °C until use.

2.2. Sample preparation

Samples were collected via venipuncture from patients with busulfan administered. After finishing the tests ordered for patient care, leftover samples (heparinized plasma) were de-identified and stored frozen at −70 °C until analysis. Sample preparation consisted of adding 300 μL internal standard to 100 μL of plasma in polypropylene microcentrifuge tubes. After vortexing for 15 s the mixture was centrifuged for 10 min at 15,500 × g. The supernatant was transferred to HPLC sample vials with 50 μL injected into the system.

2.3. Turbulent flow LC–MS/MS method

This method was developed on a Thermo Fisher TSQ Quantum Access with a Cohesive TLX2 system. Instrument software for this study consisted of Tune Master 1.5, Aria 1.6.1, and Xcalibur 2.0.7. The duplex Cohesive system consisted of a robotic sampling arm and a refrigerated sampling compartment for six 96-well plates followed by 2 parallel and independent inline degassers, binary HPLC pumps, and quaternary HPLC pumps. Samples were loaded on the Cyclone-P turbo column which was washed afterwards. Mobile phase A consisted of 50 mM ammonium formate and 0.1% formic acid while mobile phase B was 90:10 methanol:acetonitrile. At the transfer step 75 μL of mobile phase B from the transfer loop eluted the analytes from the Cyclone-P turbo column to the Hypersil GOLD analytical column where separation occurred with an isocratic elution of 70:30 mobile phase A:B. The total run time between two injections was 4.0 min if only one channel was used. The mass spectrometer was set to positive electrospray ionization mode. The spray voltage was set at 5000 V and the capillary temperature at 224 °C. The sheath gas was set to 50 U and aux gas to 25 U. Multiple reaction monitoring was set to the ammonium adduct transitions of 264.0 → 151.1 m/z for busulfan and 272.1 → 159.2 m/z for d_8-busulfan. The collision energy was 10 eV for busulfan and 11 eV for d_8-busulfan and the tube lens offset was 60 for both. Data collection started at 1.25 min into the HPLC run and continued for 2.5 min. The detail of turbulent flow and HPLC methodology is outlined in Table 1.

2.4. Validation methods

Ion suppression was evaluated by post-column infusion of either pure busulfan (4.07 μmol/L) or d_8-busulfan (3.94 μmol/L) methanol solution while supernatants of precipitated patient samples without busulfan were injected in the system (n = 5). Commercial controls (MAS Liquimmune, Camarillo, CA; Lyphochek, Irvine, CA; Monitrol, Fremont, CA) containing >100 therapeutic drugs and common endogenous substances were also extracted and analyzed to test for interference. These commercial controls were unassayed quality control materials prepared from human serum. Analyte levels were adjusted with various animal extracts and other non-protein materials including drugs, drug metabolites and purified chemicals. Linearity was examined in triplicate by serially diluting a high-concentration specimen prepared by spiking a commercial drug-free serum. Both analytical recovery and imprecision were calculated for each concentration set. Linearity was evaluated by the least square regression method with an allowable error for nonlinearity (7%) of the samples. The lowest limit of quantification was determined by the lowest concentration levels in the linearity study with accuracy within 100 ± 20% and total coefficient of variation (CV) within 20%. As per CLSI EP10-A3 guideline (Wayne, PA, USA) the sequence mid–hi–low–mid–mid–low–low–hi–hi–mid was run twice a day for 5 days using patient derived samples spiked at three levels to determine both the intra-assay and inter-assay CVs. A second low level CV was assessed using simple precision protocol (n = 20) for inter- and intra-assay. Carryover was determined by running three extractions of the sequence (low_1–high–low_2) where low_2 was a re-injection of the low_1. A passing test meant low_1 and low_2 differed by less than 20% and the low_2 was within 3 standard deviations of the low_1 value. The standard deviation was determined using low_1 values. A second carryover was per-

Table 1
LC conditions for turbo and analytical columns.

Process	Time (s)	Turbo column solvent A:B:C	Flow rate, mL/min	Analytical column solvent A:B	Flow rate, mL/min
Turbulent flow extraction on turbo column	30	98:2:0	1.5	98:2	0.65
Begin transfer from turbo to analytical column	45	98:2:0	0.1	98:2	0.65
Clean turbo column, perform separation on analytical column	15	0:0:100	1.5	70:30	0.65
Clean turbo column and valves, perform separation on analytical column	15	100:0:0	1.5	70:30	0.65
Switch valves and clean turbo column and valves, perform separation on analytical column	15	100:0:0	1.5	70:30	0.65
Clean turbo column and valves, perform separation on analytical column	15	0:100:0	1.5	98:2	0.65
Load transfer loop and clean analytical columns	30	0:100:0	1.5	98:2	0.65
Equilibrate turbo and analytical columns	75	98:2:0	1.5	98:2	0.65

Mobile phase A: 50 mM ammonium formate with 0.1% formic acid, mobile phase B: 90:10 methanol:acetonitrile, mobile phase C: organic cocktail containing 70% acetonitrile, 20% isopropanol, and 10% acetone.

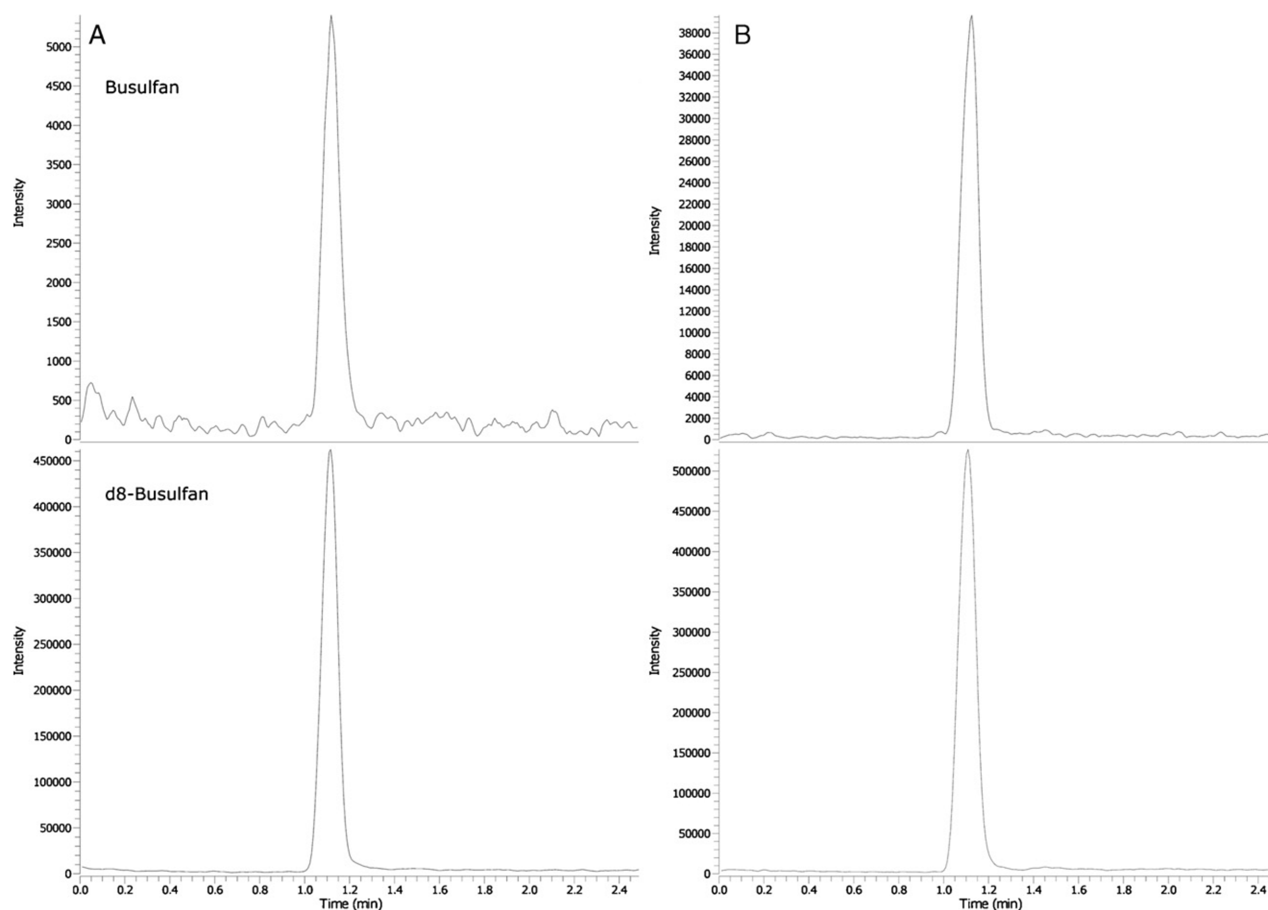

Fig. 1. Chromatograms. (A) Calibration standard with busulfan at 0.32 μmol/L and d_8-busulfan at 3.94 μmol/L; (B) A patient sample with a busulfan concentration of 1.02 μmol/L.

formed with the sequence blank–high–blank. High values above the linearity of the assay were diluted within the linear range and the value was back calculated. We tested the matrix effects of serum versus heparinized plasma by using a mixing study to determine if matrix-based interferences/suppression existed. The basis of the mixing study was to measure matrix 1, matrix 2, and matrix 1 plus matrix 2 and to determine if the measured concentrations of the mixtures matched the calculated values (differences within ±20%). As a second measure of matrix effect, serum and heparinized plasma from the same patient was spiked with the same amount of busulfan and the measured levels were compared using Deming regression for a total of 23 patients. Comparisons by Deming regression were also performed between the new LC–MS/MS assay and a well established LC–MS/MS method at Emory University Hospital [27] using 45 de-identified leftover clinical samples from patients on busulfan therapy. Statistics were calculated using Excel (Microsoft, Redmond, WA, USA) or EP Evaluator Release 8 (David G. Rhoads Associates, Kennett Square, PA, USA).

We also participated in a busulfan cross verification exercise coordinated by Otsuka American Pharmaceuticals that included seventeen institutions with eighteen results. Eight blinded quality control samples were run with theoretical values from 0 to 14.05 μmol/L. Methodologies performed varied however most were chromatography based. The criteria for acceptable laboratory performance as defined by the company were 5 out of 7 unknown samples should be within ±15% of the theoretical concentration and at least one low concentration, one intermediate concentration and one high concentration should be within ±15% of the theoretical concentration.

3. Results and discussion

The analytical cycle time was 4.0 min/injection. The retention time (mean ± SD) for busulfan and d_8-busulfan was 1.12 ± 0.01 min (n = 64) in serum or plasma samples (Fig. 1). No significant ion suppression was observed by monitoring total ion current while individually injecting supernatants of 5 precipitated patient samples with a busulfan or d_8-busulfan solution infused at 10 μL/min through a post-column T-connection prior to the mass spectrometer. Chromatographic interferences are less common with LC–MS/MS methods due to the inherent MS/MS specificity. However, a compound with the same molecular weight and ionization characteristics can possibly interfere. As such interference studies were performed using all stated commercial controls prepared as samples and injecting them into the system to determine if a non-busulfan peak appeared on the chromatogram with a retention time similar to busulfan. There was no significant interference observed from these materials.

Busulfan assay calibration was prepared at eight levels by serially diluting a high-concentration specimen prepared by spiking a commercial drug-free serum and stored at -70 °C. Quantitation was achieved based on the peak area ratios of busulfan to d_8-busulfan. Linearity was determined to be 0.15–41.90 μmol/L for busulfan by serially diluting a specimen prepared through spiking commercial drug-free serum. The lowest limit of quantification was 0.15 μmol/L based on the precision (<20%) and accuracy (100 ± 20%) in the linearity study (Table 2). IV infusion currently is the preferred means of busulfan administration and the measured levels for pharmacokinetics are above 100 ng/mL (0.41 μmol/L) [6]. Thus, an assay with a lower limit of quantification of 0.15 μmol/L is acceptable

Table 2
Precision and recovery.

Mean, μmol/L	Analytical recovery	%CV
0.06	76.5%	9.0%
0.15	91.3%	10.5%
0.28	87.9%	4.5%
0.60	93.7%	7.6%
1.20	94.5%	1.6%
2.45	96.4%	2.4%
4.94	97.3%	3.4%
9.75	95.9%	1.9%
19.24	94.6%	3.1%
41.90	103.0%	3.0%

Table 3
Precision data.

	Simple	CLSI EP10-A3 protocol		
	Low_1	Low_2	Mid	High
N	20	30	30	30
Mean, μmol/L		4.52	9.79	15.32
Total SD, μmol/L		0.34	0.51	0.79
Intra-assay SD, μmol/L	0.04	0.24	0.20	0.52
Intra-assay mean, μmol/L	0.55			
Intra-assay %CV	7.7%	5.3%	2.1%	3.4%
Inter-assay SD, μmol/L	0.05	0.24	0.47	0.60
Inter-assay mean, μmol/L	0.60			
Inter-assay %CV	7.8%	5.2%	4.8%	3.9%

for clinical use. The intra-assay and inter-assay CVs were 2.1–7.8% (Table 3) determined based on CLSI EP10-A3 guideline using patient derived samples at 3 levels and simple precision using 20 replicates of a second low patient sample. No significant carryover was observed by testing the spiked patient samples with low_1 (mean: 0.59 μmol/L)–high (41.90 μmol/L)–low_2 (0.61 μmol/L) or with blank patient samples run after a spiked high sample in the sequence $blank_1$–high (41.90 μmol/L)–$blank_2$. The mixing study ($n = 7$) showed no significantly different matrix effect between serum and heparinized plasma. Serum and heparinized plasma from the same patients ($n = 23$) were spiked with busulfan from 1.11 to 19.0 μmol/L. The Deming regression for the serum and plasma comparison showed a slope of 1.03 (95%CI: 0.98–1.07), intercept of −0.02 μmol/L (−0.59 to 0.19), a linear correlation coefficient of 0.9953, and standard error of estimate 0.53 μmol/L with a mean bias of 0.1%. Therefore, we concluded that the two specimen types can be used interchangeably.

We compared the newly developed method with a well established LC–MS/MS method [27] using 45 de-identified left-over samples (heparinized plasma) collected from patients with busulfan therapy. The values ranged from 0.94 to 17.3 μmol/L. 18 of the 45 had busulfan levels above 4.46 μmol/L, the upper linearity range by Chen et al. method [25], and would need manual dilution. The distribution of the busulfan levels was near normal distribution by visual inspection of the patient data histogram (not shown). The comparison by Deming regression between this LC–MS/MS and the Emory LC–MS/MS ($n = 45$) [27] had a slope 1.02 (95%CI: 0.97–1.06), intercept −0.02 μmol/L (−0.35 to 0.32), a linear correlation coefficient of 0.9883, and standard error of estimate 0.68 μmol/L with a mean bias of 1.3%. We have also participated in the cross verification exercise coordinated by Otsuka American Pharmaceuticals and have successfully met the passing criteria.

In conclusion, we have developed and validated a novel LC–MS/MS method using a turbulent flow online extraction technology to quantify busulfan in serum or plasma. The method is rapid, accurate, and requires little manual sample preparation.

Ethical approval

Utilizing leftover de-identified clinical samples does not need institutional review board review.

Contributors

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Conflicts of interest

No authors declared any potential conflicts of interest.

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