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Validation of a high-performance liquid chromatographic assay method for pharmacokinetic evaluation of busulfan

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Abstract

The development and validation of a high-performance liquid chromatographic (HPLC) assay for determination of busulfan concentrations in human plasma for pharmacokinetic studies is described. Plasma samples containing busulfan and 1,6-bis(methanesulfonyloxy)hexane, and internal standard, were prepared by derivatization with sodium diethyldithiocarbamate (DDTC) followed by addition of methanol and extraction with ethyl acetate. The extract was dried under nitrogen and the samples reconstituted with 100 μl of methanol prior to HPLC determination. Chromatography was accomplished using a Waters NovaPak octadecylsilyl (ODS) (150×3.9 mm I.D.) analytical column, NovaPak ODS guard column, and mobile phase of methanol–water (80:20, v/v) at a flow-rate of 0.8 ml/min with UV detection at 251 nm. The limit of detection was 0.0200 μg/ml (signal-to-noise ratio of 6) with a limit of quantitation (LOQ) of 0.0600 μg/ml for busulfan in plasma. Calibration curves were linear from 0.0600 to 3.00 μg/ml in plasma (500 μl) using a $1/y$ weighting scheme. Precision of the assay, as represented by C.V. of the observed peak area ratio values, ranged from 4.41 to 13.5% (13.5% at LOQ). No day-to-day variability was observed in predicted concentration values and the bias was low for all concentrations evaluated (bias: 0 to 4.76%; LOQ: 2.91%). The mean derivatization and extraction yield observed for busulfan in plasma at 0.200, 1.20 and 2.00 μg/ml was 98.5% (range 93.4 to 107%). Plasma samples containing potential busulfan metabolites and co-administered drugs, which may be present in clinical samples, provided no response indicating this assay procedure is selective for busulfan. This method was used to analyze plasma concentrations following administration of a 1 mg/kg oral busulfan dose.

Keywords: Busulfan

1. Introduction

Busulfan is a bifunctional alkylating agent frequently used in combination with other agents as a preparative regimen for bone marrow transplantation procedures. Patients receiving high-dose busulfan

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therapy exhibit significant non-hematologic toxicity, especially veno-occlusive disease of the liver [1–9]. Initial pharmacodynamic studies have suggested a relationship between the severity of hepatotoxicity and busulfan pharmacokinetic parameters [4,10,11] indicating that first-dose busulfan pharmacokinetics may enable optimization of therapy with this regimen [12,13].

Chromatographic methods for busulfan analysis reported in the literature include HPLC [7,14–19], GC–MS [20,21] and GC with electron-capture detection (ECD) [15,22–25]. The sensitivity and reproducibility necessary to evaluate busulfan in clinically relevant samples has been demonstrated by both GC–MS [9,20,21] and GC-ECD [22–27] techniques.

The available HPLC methods, however, are less suitable for routine analysis of busulfan. Hassan and Ehrsson [15,16] developed reversed-phase HPLC assay methods to evaluate the in vitro and in vivo degradation of busulfan but a radioactive label was required for detection. HPLC quantitation of busulfan was also described by Blanz et al. [14] using post-column derivatization in a handknitted reactor which complicates implementation of this assay in other laboratories for routine analysis. Henner et al. [17] described a pre-column diethyldithiocarbamate (DDTC) derivatization HPLC method for determination of busulfan in plasma which provided similar sensitivity and convenience to the established GC procedures. This derivatization method has been used for busulfan determination in patient samples [7]. However, one group [19] found endogenous compounds which interfered with busulfan quantitation. MacKichan and Bechtel [18] subsequently improved this assay via addition of an internal standard and modification of the sample handling procedure.

Assay methods are required for investigating the pharmacokinetic–pharmacodynamic relationships of busulfan in order to understand and optimize therapy in this patient population. To date, suitable HPLC methods for determination of busulfan in human plasma have not been described. This report described optimization and validation of an HPLC assay method using pre-column DDTC derivatization for quantitation of busulfan in human plasma. Sample handling and chromatographic run times were minimized to provide quantitative results promptly while maintaining the sensitivity, specificity, accuracy and precision required for pharmacokinetic evaluation of busulfan in the clinical setting. Furthermore, a busulfan analogue which is extracted and derivatized along with busulfan was incorporated into the assay procedure as internal standard.

2. Experimental

2.1. Materials

HPLC grade ethyl acetate and methanol were obtained from Fisher Scientific (Vancouver, Canada). HPLC grade water was prepared fresh daily with the Milli-Q system (Millipore, Bedford, MA, USA). Busulfan, tetrahydrothiophene 1-oxide, sulfolane, 1,6-hexanediol and sodium DDTC were obtained from Aldrich (Milwaukee, WI, USA). Millipore HV filters (pore size, 0.45 μm) (Millipore, Bedford, MA, USA) were used for filtration of the HPLC mobile phase just prior to use.

The following drugs were obtained from the supplier indicated to evaluate assay specificity: Carboplatin injection (10 mg/ml) (Faulding, Vaudreuil, Canada); heparin injection (10 000 IU/ml) (Organon Teknika, Scarborough, Canada); diazepam (5 mg tablet) (Hoffman La Roche, Mississauga, Canada); prochlorperazine injection (5 mg/ml) (Sabex, Boucherville, Canada); ondansetron injection (2 mg/ml) (Glaxo, Mississauga, Canada); lorazepam injection (4 mg/ml) (Wyeth-Ayerst, St. Laurent, Canada); cyclosporine IV (50 mg/ml) (Sandoz, Whitby, Canada); methylprednisolone (4 mg tablet) (Upjohn, Don Mills, Canada); methotrexate sodium (2.5 mg tablet) (Lederle Cyanamid, Markham Canada); methotrexate sodium (2.5 mg tablet) (Lederle Cyanamid, Markham, Canada); pentoxifylline (40 mg tablet) (Hoechst-Roussel, Montreal, Canada); allopurinol (100 mg tablet) (Burroughs Wellcome, Kirkland, Canada); phenytoin injection (50 mg/ml) (Parke-Davis, Scarborough, Canada).

2.2. Instrumentation

2.2.1. HPLC

Specificity was evaluated on a Hewlett Packard 1050 HPLC system with a variable wavelength UV

detector, Model 3396A integrator (Hewlett Packard, Avondale, PA, USA) and Rheodyne 7125 injector (Alltech Associates, Deerfield, IL, USA). The HPLC system used for the remainder of the work described herein consisted of a Waters 710B autosampler, a 510 solvent delivery system, a 486 variable wavelength detector at 251 nm and the Millennium Version 2.10 software used on a NEC Image 466es computer (Waters Associates, Milford, MA, USA). Analysis was performed on a NovaPak ODS (150× 3.9 mm I.D.) analytical column with a NovaPak ODS guard column (Waters Associates). The mobile phase consisted of methanol–water (80:20, v/v) at a flow-rate of 0.8 ml/min.

2.2.2. HPLC–MS and MS

The HPLC–MS system was composed of a Hewlett Packard Model 1090 Series II liquid chromatograph (Hewlett Packard), coupled to a VG Quattro Quadropole MS (Fisons, Altrincham UK) operated in the positive electrospray ionization mode. During scan mode, MS conditions included a source temperature of 120°C, a cone voltage of 22–26 V, and relative high mass/low mass resolution values of 12.5/12.5. The chromatographic conditions used were as described for the HPLC–UV method. Following chromatography, the flow was split with one-tenth of the chromatographic eluent entering the MS. Mass spectra were obtained by scanning from m/z 100 to m/z 500 at 200 mass units/s. For MS evaluation, samples were introduced directly into the MS.

2.2.3. Equipment

Sample processing required the following: Silencer H-103N centrifuge (Western Scientific Services, Richmond, Canada); Vortex Genie (Fisher Scientific, Fairlawn, NJ, USA); Labquake Shaker rotators (Labindustries, Berkeley, CA, USA); Reacti-Vap drying apparatus (Pierce, Rockford, IL, USA).

2.3. Synthesis of 3-hydroxysulfolane and 1,6-bis(methanesulfonyloxy)hexane

3-Hydroxyfulfolane was synthesized according to Onkenhout et al. [28] as described previously [24]. 1,6-Bis(methanesulfonyloxy)hexane was synthesized from 1,6-hexanediol for use as internal standard according to Embree et al. [24].

2.4. Synthesis of 1,4-bis(diethyldithiocarbamoyl)butane

1,4-Bis(diethyldithiocarbamoyl)butane, the DDTC derivative of busulfan was synthesized according to Henner et al. [17] without modification. A solution of 0.7 mg of the DDTC derivative of BU was dissolved in 1 ml of HPLC grade methanol and a portion (2 μl) was evaluated using MS and HPLC–MS. A melting point of 76–88°C was observed (literature m.p. 79–80°C [17]).

2.5. Preparation of standard solutions and reagents

Busulfan (10 mg) was accurately weighted, dissolved in HPLC grade ethyl acetate, made up to volume in a 100 ml flask and mixed. Serial dilutions in HPLC grade ethyl acetate were made to the following final concentrations: 1.50, 2.50, 5.00, 15.0, 25.0, 30.0, 40.0, 50.0 and 75.0 μg/ml for use as the busulfan working solutions. Similarly, a solution of 1,6-bis(methanesulfonyloxy)hexane (40 μg/ml) was prepared in HPLC grade ethyl acetate for use as internal standard. All the busulfan and internal standard solutions were stored at 4°C. The derivatization reagent solution was prepared just prior to use by dissolving DDTC (0.82 g) in HPLC grade water (10 ml). Calibration curve samples were prepared by adding 20 μl of the final busulfan working solutions to plasma (0.5 ml). For evaluation of the limit of detection, the busulfan working solutions at 1.50, 2.50 and 5.00 μg/ml were diluted 1:10 with HPLC grade ethyl acetate to provide concentrations of 0.150, 0.250 and 0.500 μg/ml.

2.6. Derivatization and extraction of plasma

The internal standard solution (20 μl) was added to plasma (0.5 ml) in 100×16 mm screw-capped PTFE-lined glass tubes. Following the addition of 125 μl of DDTC reagent, each tube was vortex mixed; HPLC grade methanol (0.2 ml) and ethyl acetate (2 ml) were then added. The tube was capped, vortex-mixed for 10 s and mixed by rotation

for an additional 5 min. Samples were centrifuged for 10 min at 600 *g*, the organic phase transferred to clean 100×16 mm disposable glass tubes and dried under nitrogen. The dried extract was reconstituted with methanol (100 μl), vortex-mixed, transferred to a clean autosampler vial and injected into the HPLC (30 μl).

2.7. Assay validation

For HPLC–MS confirmation of the derivatization procedure for busulfan and the internal standard in plasma, an aqueous sample containing 12 μg/ml busulfan and 80 μl internal standard was prepared, dried under nitrogen, reconstituted in 100 μl of mobile phase and evaluated using HPLC–MS (10 μl injection).

Plasma calibration curves, each consisting of one blank plus nine samples containing busulfan, were prepared and assayed in quadruplicate on three occasions to evaluate linearity, precision and accuracy.

The recovery of busulfan from both the derivatization and extraction procedures was evaluated at busulfan concentrations of 0.200, 1.20 and 2.00 μg/ml in plasma. Recovery was determined by comparison of peak areas for busulfan in plasma (derivatized and extracted) ($n=5$) to derivatized and extracted drug free plasma samples to which derivatized busulfan (equivalent to busulfan concentrations of 0.200, 1.20 and 2.00 μg/ml) was added just prior to analysis ($n=5$). The limit of detection was evaluated by analysis of plasma samples containing 0.00600, 0.0100 and 0.0200 μg/ml busulfan in quadruplicate. Stability of derivatized busulfan and internal standard was assessed by preparation and analysis of five plasma samples containing 3.00 μg/ml busulfan on one day followed by evaporation of the methanol, storage of the partially dried sample at room temperature for 24 h followed by reconstitution with methanol (100 μl) and re-injection into the HPLC.

Potential assay interference from busulfan metabolites, endogenous compounds and co-administered drugs was evaluated by analysis of drug-free plasma from five individuals and plasma containing the following compounds: 3-hydroxysulfolane (0.1 μg/ml), sulfolane (0.1 μg/ml), tetrahydrothiophene 1-oxide (0.1 μg/ml), ondansetron (1.0 μg/ml), carboplatin (2 μmol/l), phenytoin (40 μg/ml), prochlorperazine (1.0 μg/ml), lorazepam (0.26 μg/ml), heparin (0.9 units/ml), cyclosporine (32.5 μg/ml), diazepam (0.63 μg/ml), methylprednisolone (5.3 μg/ml), methotrexate (3.3 μg/ml), pentoxifylline (51.5 μg/ml) and allopurinol (0.40 mg/ml).

2.8. Clinical samples

Two patients undergoing treatment with a busulfan containing preparative regimen (1 mg/kg orally every 6 h for 16 doses) gave informed consent to this study which was approved by the University of BC Ethics Committee and the local Hospital ethics review boards. Blood samples were collected in ethylene diaminetetraacetic acid (EDTA) Vacutainer tubes (Becton Dickinson, Mississauga, Canada) just prior to and 15, 30, 60, 90, 120, 180, 240 and 360 min following administration of the first busulfan dose. In addition, samples were collected just prior to and at 1, 2 and 6 h after busulfan dose 9. All samples were kept at 4°C, centrifuged within 6 h of collection and the plasma separated and stored at −20°C until analysis. Plasma samples were analyzed by the HPLC assay procedure described above.

3. Results and discussion

Our modifications to the previously reported HPLC assay methods [17,18] primarily involve elimination of endogenous interference by optimizing the analytical column stationary phase, development of a simplified sample handling procedure for determination of busulfan at therapeutically relevant concentrations, and inclusion of 1,6-bis(methanesulfonyloxy)hexane as internal standard. Endogenous plasma components reported to co-elute with the DDTC derivative of busulfan were eliminated by use of NovaPak ODS rather than μBondapak ODS as stationary phase. The solid-phase extraction sample clean-up described by MacKichan and Bechtel [18] was not required with the NovaPak ODS stationary phase, thereby greatly simplifying the required sample handling. In addition, the internal standard previously used [18] was extracted from plasma but did not undergo derivatization and therefore would

be unable to account for any sample to sample changes in the overall plasma derivatization and extraction procedure. We have synthesized the 1,6 analogue of busulfan 1,6-bis(methanesulfonyloxy)-hexane) for use as internal standard in the assay reported here as it is both derivatized and extracted along with busulfan during sample preparation. The structure of busulfan, the internal standard and their DDTC derivatives is shown in Fig. 1. Representative chromatograms of blank plasma and plasma containing busulfan plus samples obtained from a patient receiving busulfan as preparative therapy for bone marrow transplantation are shown in Fig. 2. These chromatograms demonstrate that endogenous components of both drug free plasma and the patient plasma elute prior to the derivatized busulfan and derivatized internal standard.

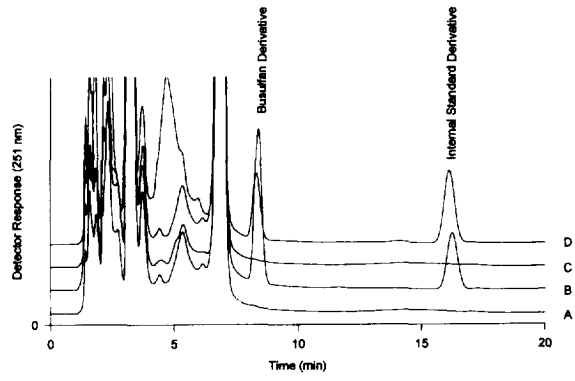

Fig. 2. Chromatograms of (A) blank plasma; (B) plasma containing 3.00 μg/ml busulfan; (C) pre-treatment patient plasma; (D) a sample from the same patient taken 2 h post-administration of busulfan dose 9.

3.1. HPLC–MS and MS confirmation

MS of the large scale busulfan derivatives from m/z 100 to m/z 500 provided an MH^+ ion for 1,4-bis(diethyldithiocarbamoyl)butane (M_r=352.1) at m/z 353 and its sodium adduct at m/z 375. HPLC–MS demonstrated that the MH^+ ion and its sodium adduct co-eluted at 8.34 min with the chromatographic and detection conditions used. An impurity was also detected at m/z 269 (base mass) which eluted at 1.42 min using HPLC prior to MS and was

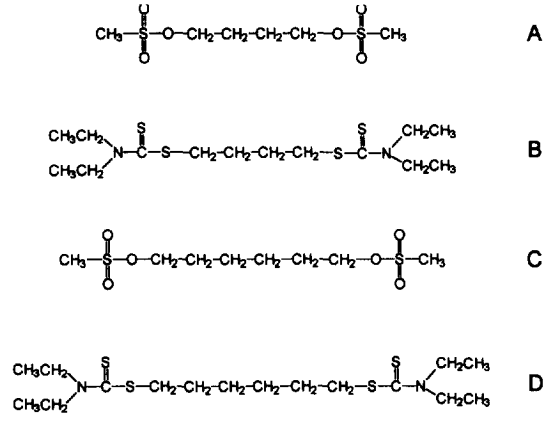

Fig. 1. Structure of (A) busulfan; (B) 1,4-bis(diethyldithiocarbamoyl) butane, the DDTC derivative of busulfan; (C) the internal standard 1,6-bis(methanesulfonyloxy)hexane; (D) its DDTC derivative.

therefore resolved from the busulfan derivative. Ions seen at m/z 116 and m/z 204 eluted at 8.34 min are fragments of the MH^+ ion at m/z 353 (as confirmed by a daughter scan of m/z 353) and are consistent with the proposed structure of this busulfan derivative (see Fig. 1).

HPLC–MS analysis of the small scale (Plasma) derivatization procedure for busulfan verified that the proposed derivative of busulfan was formed by this method. At the retention time of the internal standard DDTC derivative (M_r=380.1) (16.5 min), the MH^+ ion at m/z 381 and fragment ions at m/z 116 and m/z 232 confirm the proposed structure shown in Fig. 1.

3.2. Recovery

The efficiency of the derivatization and extraction procedure for busulfan in plasma was determined by comparison of the peak areas observed for samples at 0.200, 1.20 and 2.00 μg/ml, respectively. This recovery of busulfan from plasma is similar to that observed previously with the solid-phase extraction procedure [18]. The recovery from the large scale derivatization conducted at 50°C provided a significantly lower recovery [17,19] than that observed here for plasma samples. Loss during the purification process used for larger samples may, in part, account for this difference.

Table 1
Precision data for HPLC assay of busulfan in plasma

Concentration (μg/ml)	Plasma Peak Area Ratio (busulfan/internal standard)		
	Mean	C.V. (%)	*n*
0.0600	0.0335	13.5	12
0.100	0.0635	9.60	11
0.200	0.121	10.6	12
0.600	0.381	5.46	11
1.00	0.625	5.70	11
1.20	0.763	4.41	10
1.60	1.00	5.31	11
2.00	1.25	6.77	12
3.00	1.89	6.31	11

3.3. Calibration curves, precision, accuracy and linearity

Precision of the peak area ratio values, determined from the validation experiment data, is provided in Table 1. The % coefficients of variation (C.V.) were below 15% (range 4.41–13.5%; LOQ 13.5%). Regression analysis of the validation data demonstrated that a $1/y$ weighted linear relationship between peak area ratio versus busulfan concentration described the calibration curve for busulfan in plasma. An overall correlation coefficient of 0.9989 was obtained for the twelve curves (range 0.9967–1.0000). To examine the ability of the calibration curves to predict concentration values, predicted concentration values for three of the four curves evaluated each day were calculated using the remaining curve from that day. In this manner, a total of four values were predicted for each concentration for each day (total of twelve values per concentration). The statistical summary of the predicted values is given in Table 2.

Table 2
Statistics for predicted busulfan concentrations in plasma

Actual concentration (μg/ml)	Mean (μg/ml)	Bias (μg/ml)	S.D. (μg/ml)	C.V. (%)
0.0600	0.0618	0.0018	0.0126	20.4
0.100	0.105	0.005	0.0122	11.6
0.200	0.197	−0.003	0.0235	11.9
0.600	0.607	0.007	0.0272	4.48
1.00	0.995	−0.005	0.0501	5.04
1.20	1.22	0.02	0.0469	3.84
1.60	1.61	0.01	0.0676	4.20
2.00	2.00	0	0.104	5.20
3.00	3.02	0.02	0.192	6.36

One-way analysis of variance indicated that there was no significant difference in the predicted levels on the three different days. Precision of the predicted values, as represented by the C.V. values, ranged from 3.84–20.4% (at LOQ 20.4%). The bias was low for all concentration values (bias 0 to 4.76%; LOQ 2.91%). The calibration curve data demonstrates the linearity of the assay procedure over the concentration range 0.0600–3.00 μg/ml plasma.

3.4. Limits of detection and quantitation

The limit of detection was determined by evaluation of plasma samples containing from 0.00600 to 0.0200 μg/ml busulfan in quadruplicate. The lowest detectable amount of busulfan was found to be 0.0200 with a signal-to-noise ratio of 6. The limit of quantitation was determined as the lowest evaluable concentration demonstrating a precision (peak area ratio C.V.) and accuracy (bias) of less than 15%. A limit of quantitation of 0.0600 μg/ml was observed for this assay.

3.5. Specificity

The specificity of the assay procedure was evaluated by investigation of the response produced by potential busulfan metabolites and co-administered drugs which may be present in plasma from patients receiving busulfan preparative therapy prior to bone marrow transplantation. The assay procedure provided no response to the potential busulfan metabolites sulfolane, tetrahydrothiophene 1-oxide and 3-hydroxysulfolane in plasma. The following drugs also demonstrated no response with this assay method: phenytoin, heparin, lorazepam, diazepam, methotrexate, cyclosporine, methylprednisolone, carboplatin, ondansetron, prochlorperazine, pentoxifylline and allopurinol. No interference was observed from endogenous compounds present in the drug-free plasma from five individuals. Endogenous components in human plasma have previously been evaluated as potential sources of interference with DDTC pre-column derivatization procedures for busulfan [7,17–19]. Indeed, Kazemifard and Morgan [19] were unable to remove endogenous interference from drug free plasma. This is the first report describing the lack of interference from potentially co-administered drugs or busulfan metabolites.

3.6. Clinical samples

Plasma samples from two patients were evaluated with the assay procedure described above. Representative chromatograms obtained on analysis of the pre-treatment sample (patient blank) and the sample obtained 2 h following dose 9 from one patient are shown in Fig. 2. The range of busulfan concentration values for both patients was found to be from 0 to 1.90 μg/ml which is well within the linear range of this assay procedure.

4. Conclusions

The assay method described for HPLC determination of busulfan meets the requirements for pharmacokinetic studies [29] in addition to the time constraints on assay methods to be useful for therapeutic monitoring and pharmacokinetic studies. As demonstrated by the plasma data obtained to date from our patients and those in our previous pharmacokinetic study [30–32], this method is suitable for evaluation of busulfan pharmacokinetic behaviour when administered as part of bone marrow transplantation preparative regimes.

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