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A gas-chromatographic assay method for busulfan with sensitivity for test dose therapeutic monitoring

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Abstract

A gas-chromatographic assay method was developed and validated for determination of busulfan in human plasma for test dose therapeutic drug monitoring. Busulfan and the internal standard (1,6-bis-(methanesulfonyloxy)hexane) were extracted from plasma samples and derivatized with 2,3,5,6-tetrafluorothiophenol prior to gas chromatographic determination. The ^{63}Ni electron-capture detector provided a limit of quantitation of 0.0100 μg ml^{-1} busulfan in plasma with a linear response over the concentration range 0.0100–0.400 μg ml^{-1}. Extraction and derivatization yields were 85.3%–91.0% and greater than 95%, respectively. Assay specificity for busulfan in the presence of potential metabolites was demonstrated. Potentially co-administered drugs gave no response under the sample preparation and chromatographic conditions described for quantification of busulfan. The applicability of this assay to the individualization of busulfan therapy based on a 2 mg test dose is discussed.

Keywords: Busulfan; GC/ECD; Test dose; Therapeutic drug monitoring

1. Introduction

Busulfan is a bifunctional alkylating agent currently in use at high doses (16 mg kg^{-1}) in preparative chemotherapeutic regimes for bone marrow transplantation procedures. Significant toxicities, in particular veno-occlusive disease of the liver [1–11], have been observed with high dose busulfan therapy. A correlation between the occurrence of this toxicity and busulfan pharmacokinetics increased exposure as determined by area under the plasma-concentration versus time curve) has been demonstrated [4,12,13], and has prompted investigations into individualizing busulfan dosing based on monitoring first-dose pharmacokinetics [14–16].

Therapeutic drug monitoring with individualized busulfan therapy using first-dose pharmacokinetics has potential complications [17]. Individualized therapy is not possible until the second or subsequent doses, owing to the necessity of obtaining samples from the first dose for evaluation. This requires immediate evaluation of the busulfan samples which places time constraints on data analysis [14]. Venous access, emesis and variable absorption further complicate individualized dosing when first-dose pharmaco-kinetics is used [17,18].

A test dose of a drug to evaluate patient response can be administered before initiation

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of therapy. Use of a test dose for determining individual pharmacokinetic behavior prior to therapeutic dosing has been proposed for busulfan [17]. Potential advantages of evaluating a test dose include minimization of the above-described complications and the ability to use an individualized dose upon initiation of therapy. In addition, off-site drug quantification and pharmacokinetic evaluation become possible.

Chromatographic methods for busulfan analysis include high-performance liquid chromatography (HPLC) [8,19–22], gas chromatography/mass spectrometry (GC/MS) [23,24] and GC with electron-capture detection (ECD) [20,25–27]. Routine analysis of busulfan with the HPLC methods described in the literature is complicated by their poor sensitivity, the presence of interfering peaks [22,28] or the necessity for a radioactive label [20,21]. The GC/MS procedures provide sufficient sensitivity for evaluation of a test dose [23,29] but have costly equipment requirements. GC/ECD methods reported to date have not provided the desired sensitivity to evaluate busulfan pharmacokinetics following administration of a 2 mg test dose.

This report describes the validation of a sensitive GC/ECD assay method using 2,3,5,6-tetrafluorothiophenol (TFTP) derivatization, with sufficient sensitivity to quantify busulfan in plasma after administration of a 2 mg test dose.

2. Materials and methods

2.1. Gas chromatography

A model 5890 Hewlett-Packard gas chromatograph (Hewlett-Pachard, Avondale, PA), equipped with a ^{63}Ni (15 mCi) ECD was employed. Analysis was performed on a Supelco 2250 fused silica capillary column (15 m × 0.32 mm i.d.) with a film thickness of 0.20 μm (Supelco, Belleforte, PA) using previously reported chromatographic conditions [27].

2.2. Equipment

Sample processing required the following equipment: Silencer H-103N centrifuge (VWR Scientific, London, Ont., Canada); Vortex Genie (Fisher Scientific, Fairlawn, NJ, USA); Vortex-Evaporator heating block (Buchler Instruments, Fort Lee, NJ, USA); Labquake Shaker rotators (Labindustries, Inc., Berkeley, CA, USA); Reacti-Vap drying apparatus (Pierce Chemical Co., Rockford, IL, USA).

2.3. Materials

Busulfan, sulfolane, tetrahydrothiophene-1-oxide, and TFTP were obtained from Aldrich (Milwaukee, WI, USA). Methanol, ethyl acetate and hexane purchased from Fisher Scientific Co. (Fairlawn, NJ, USA) were HPLC grade. 1,6-Bis(methanesulfonyloxy)hexane for use as internal and external standard was synthesized as described by Embree et al. [27]. The following chemicals were also used: 3-sulfolane (Eastman Organic Chemicals, Rochester, NY, USA); carboplatin (Bristol Laboratories, Belleville, Ont., Canada); phenytoin (Smith & Nephew, Lachine, Que., Canada); cyclophosphamide (Horner, Montreal, Que., Canada); cytarabine (Upjohn Co., Don Mills, Ont., Canada); and sodium hydroxide (Aristar grade, BDH Chemicals Ltd., Poole, UK)

2.4. Preparation of calibration standards and reagents

Busulfan (100 mg) was accurately weighed, dissolved in ethyl acetate, made up to volume in a 100 ml flask and mixed. Serial dilutions of this stock solution were prepared in ethyl acetate to final concentrations of 1.50, 2.50, 5.00, 12.5, 25.0 and 30.0 μg ml^{-1} for use as working solutions. An internal standard solution of 40 μg ml^{-1} was prepared in ethyl acetate for quantification of busulfan and for use as external standard in the extraction recovery studies. The derivatization reagent solution was prepared just prior to use by mixing TFTP (0.040 ml) and methanol (2 ml). Sodium hydroxide (1 M) in HPLC grade water was prepared on a monthly basis. Seven calibration curve samples were prepared by adding 10 μl of the 1.50 μg ml^{-1} busulfan working solution 20 μl of all six busulfan working solutions to drug-free plasma (1.5 ml), to provide final concentrations of 0.0100, 0.0200, 0.0333, 0.0667, 0.167, 0.333 and 0.400 μg ml^{-1} in plasma.

2.5. Synthesis of TFTP derivatives

Synthesis of the TFTP derivatives of busulfan and the internal standard has been previously described [27].

2.6. Extraction and derivatization

A modified extraction and derivatization procedure was developed by modification of the methods reported by Chen et al. [25] and Embree et al. [27]. Internal standard (20 μl) was added to plasma (1.5 ml) in a 16 × 100 screw-capped PTFE-lined glass tube. Following the addition of ethyl acetate (4 ml), the tube was capped, vortex-mixed for 10 s and rotated for an additional 10 min. The sample was centrifuged for 10 min at 1000*g*, and the organic phase transferred to a clean 16 × 100 mm screw-capped PTFE-lined tube and dried under nitrogen. Water (HPLC grade; 200 μl), freshly prepared derivatization reagent solution (20 μl) and acqueous sodium hydroxide (20 μl) was added. The sample was then capped, vortex-mixed for 10 s and heated at 70 °C for 30 min. After derivatization, sodium hydroxide solution (1 ml) and hexane (500 μl) were added to the sample, followed by vortex-mixing for 10 s, rotating for 10 min and centrifuging for 10 min at 1000*g*. The organic phase was transferred to a clean vial and injected into the gas chromatograph (1 μl).

2.7. Assay validation

Plasma calibration curves, each consisting of seven samples containing busulfan (from 0.0100 to 0.400 μg ml^{-1}) and internal standard, were prepared and assayed in quadruplicate on three separate occasions.

The limit of quantification of this assay method was determined by evaluation of four separate samples of busulfan in plasma at a concentration of 0.0100 μg ml^{-1} on three occasions. Busulfan recovery from plasma was determined by comparison of peak ratios (busulfan/external standard) of extracted to unextracted samples at busulfan concentrations of 0.0100, 0.0667 and 0.400 μg ml^{-1} plasma ($n = 3$ for each concentration). Comparing the busulfan peak areas for the unextracted samples with peak areas obtained from samples containing equimolar amounts of the TFTP busulfan derivative ($n = 10$ for each concentration) provided the efficiency of the derivatization procedure.

Potential assay interference from busulfan metabolites, endogenous compounds and co-administered drugs was evaluated by analysis of drug-free plasma from three individuals and the following compounds in plasma: 3-hydroxysulfolane (0.1 μg ml^{-1}), sulfolane (0.1 μg ml^{-1}), tetrahydrothiophene-1-oxide (0.1 μg ml^{-1}), ondansetron (100 ng ml^{-1}), carboplatin (2 μmol ml^{-1}), phenytoin (20 μg ml^{-1}), cyclophosphamide (10 μmol ml^{-1}) and cytarabine (5.0 μM).

3. Results

3.1. Recovery

The efficiency of ethyl acetate extraction of busulfan from plasma was determined by comparison of the peak area ratios (busulfan/external standard) for extracted and unextracted samples at concentrations of 0.0100, 0.0667 and 0.400 μg ml^{-1} plasma. Busulfan extraction efficiencies, expressed as mean ± SD, were 88.1 ± 10%, 85.3 ± 7.5% and 91.0 ± 9.4% for the 0.0100, 0.667 and 0.400 μg ml^{-1} samples, respectively. The derivatization yield for busulfan standards in plasma at concentrations of 0.0100, 0.0667 and 0.400 μg ml^{-1}, estimated by comparison of the peak areas from unextracted busulfan samples with those from direct injection of equimolar amounts of the TFTP busulfan derivative, were 97.0 ± 15%, 92.0 ± 6.4% and 101 ± 3.9%, respectively.

3.2. Specificity, ruggedness, precision and limit of quantification

The assay procedure demonstrated no response to potential busulfan metabolites (sulfolane, tetrahydrothiophene-1-oxide and 3-hydroxysulfolane) and potentially co-administered drugs (phenytoin, carboplatin, ondansetron, cytarabine and cyclophosphamide) in plasma.

One-way analysis of variance demonstrated significant day-to-day variability for area ratio values at five of the seven busulfan concentrations evaluated. Precision data for the assay procedure, determined using a total of 12 samples at each concentration from the calibration curve validation experiment, are listed in Table 1. The RSD ranged from 2.2 to 13%, with a mean of 7.0%. The limit of quantification for busulfan in plasma was found to be 0.0100 μg ml^{-1}, which provided acceptable precision and accuracy. Chromatograms obtained from analysis of plasma samples with and without addition of busulfan are shown in Fig. 1.

Table 1
Precision of GC/ECD assay for busulfan in plasma

Concentration ($\mu g\ ml^{-1}$)	Mean peak area ratio ($n = 12$)	Standard deviation	RSD (%)
0.0100	0.0323	0.00199	6.2
0.0200	0.0528	0.00686	13
0.0333	0.0877	0.00783	8.9
0.0667	0.187	0.0129	6.9
0.167	0.462	0.0159	3.4
0.333	0.977	0.0843	8.6
0.400	1.17	0.0261	2.2

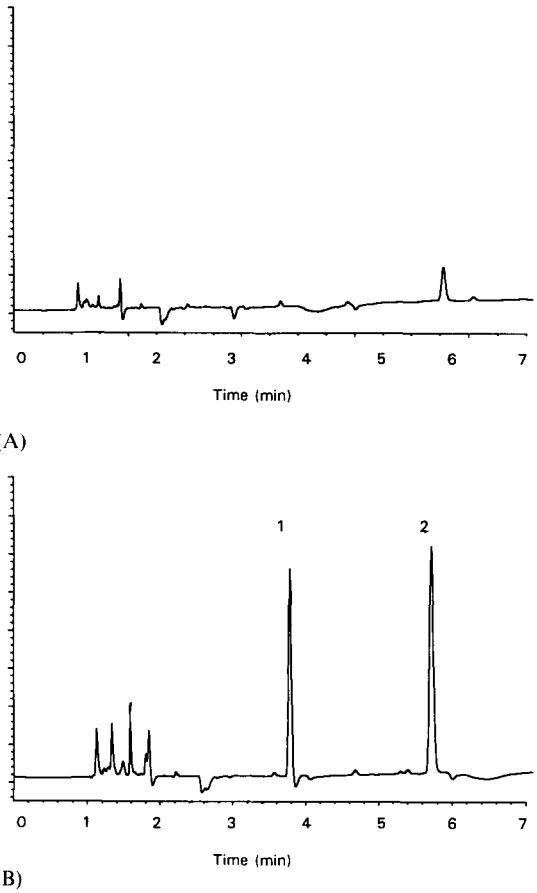

Fig. 1. Chromatograms of extracted and derivatized (A) blank plasma and (B) 0.400 $\mu g\ ml^{-1}$ busulfan in plasma. Peaks: (1) busulfan TFTP derivative; (2) internal standard derivative.

3.3. Calibration curves

Regression analysis of the calibration curve validation data demonstrated that Eq. (1), relating the area ratio and busulfan concentration, described the calibration curves for busulfan. The 12 calibration curves gave correlation coefficients from 0.9924 to 0.9998, with an overall correlation coefficient of 0.9971.

$$\text{Area ratio} = b_0 + b_1(\text{concentration}) \quad (1)$$

The calibration curve validation data was divided into four calibration curves for each day to examine the ability of the calibration curve to predict busulfan concentration values. Predicted values for three curves were calculated using the remaining curve from that day, so that a total of four values were predicted for each concentration for each day (a total of 12 values per concentration per day). Table 2 describes the statistical summary of the predicted values obtained in this manner. One-way analysis of variance indicated that the predicted levels on the three days were not significantly different. Good agreement between the actual and predicted concentrations demonstrated the accuracy of this assay procedure.

4. Discussion

Modifications to a previously published GC/ECD assay [27] involved optimazation of the extraction and derivatization procedures for the quantification of busulfan in plasma under clinically relevant concentrations following a 2 mg test dose. Increased sensitivity of the assay was primarily accomplished by improving the extraction efficiency and derivatization yield, and by increasing the plasma sample volume.

Analysis of calibration curves demonstrates assay linearity over the concentration range studied (0.0100–0.400 $\mu g\ ml^{-1}$ plasma) with correlation coefficients of greater than 0.99. No significant day-to-day variability was observed for the predicted concentrations. A limit of quantification of 0.0100 $\mu g\ ml^{-1}$ in plasma was observed, and is approximately ten-fold lower than previously described [27] for GC/ECD. Using GC/MS for quantification of busulfan, a

Table 2
Accuracy of GC/ECD assay for busulfan in plasma

Actual concentration ($\mu g\ ml^{-1}$)	Mean ($n = 36$)	Bias (%)	Standard deviation	RSD (%)
0.0100	0.0109	+0.0009 (8.26)	0.00201	18
0.0200	0.0196	−0.0004 (2.04)	0.00308	16
0.0333	0.0317	−0.0016 (5.05)	0.00349	11
0.0667	0.0674	+0.0007 (1.04)	0.00620	9.2
0.167	0.159	−0.008 (5.03)	0.0146	9.2
0.333	0.327	−0.006 (1.83)	0.0241	7.4
0.400	0.399	−0.001 (0.251)	0.0423	11

limit of quantification of 0.0100 $\mu g\ ml^{-1}$ plasma was sufficient for pharmacokinetic studies following 2 mg doses [23,29]. Therefore, the assay method described here also provides the sensitivity required for analysis of busulfan after administration of a 2 mg dose.

The assay method described herein for GC/ECD determination of busulfan in plasma meets the requirements for pharmacokinetic studies [30]. This method provides the means to evaluate the potential relationships between test dose pharmacokinetics and both therapeutic dose pharmacokinetics and toxicity. Using the test dose for pharmacokinetic evaluation and individualization of busulfan therapy would allow dose modification at intitiation of therapeutic doses, and would maximize the benefits of therapeutic drug monitoring.

5. Conclusions

The utility of 2 mg test dose pharmacokinetics for providing individualized busulfan dosing in preparative chemotherapeutic regimens for bone marrow transplantation needs to be investigated. Validation data reported here and previously established pharmacokinetic behavior of busulfan [23,29] demonstrate that the assay method described herein is suitable for evaluation of busulfan following administration of 2 mg doses. This assay has improved the sensitivity of GC/ECD quantification of busulfan to that observed for GC/MS.

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