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Gas-chromatographic analysis of busulfan for therapeutic drug monitoring

Leanne Embree[1, 2], Robbin B. Burns[2], Jean R. Heggie[1], Gordon L. Phillips[3], Donna E. Reece[3], John J. Spinelli[3], Daria O. Hartley[1], Norma J. Hudon[1], James H. Goldie[1, 4]

[1] Division of Medical Oncology, Section of Laboratory Operations, British Columbia Cancer Agency, Vancouver, B. C., Canada
[2] Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, B. C., Canada
[3] Leukemia and Bone Marrow Transplantation Program of British Columbia, Division of Hematology, British Columbia Cancer Agency, Vancouver General Hospital and Faculty of Medicine, University of British Columbia, Vancouver, B. C., Canada
[4] Division of Medical Oncology, British Columbia Cancer Agency and Faculty of Medicine, University of British Columbia, Vancouver, B. C., Canada

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Abstract. The development and validation of a gas chromatographic assay method for determination of total and free busulfan concentrations in human plasma for pharmacokinetic studies is reported. 1,6-*Bis*(methanesulfonyloxy)hexane, the internal standard, and a potential metabolite, 3-hydroxysulfolane, were synthesized. Plasma and plasma ultrafiltrate samples containing busulfan and internal standard were extracted with ethyl acetate and derivatized with 2,3,5,6-tetrafluorothiophenol prior to gas chromatographic determination. The ^{63}Ni electron-capture detector provided a limit of detection of 0.0600 μg/ml with a limit of quantitation of 0.100 μg/ml busulfan in biological samples. Calibration curves were linear from 0.100 to 3.00 μg/ml in plasma (500 μl) and 0.100 to 2.00 μg/ml in plasma ultrafiltrate (100 μl). Extraction and derivatization yields ranged from 78.4% to 89.6% and 56.0% to 71.3%, respectively. Specificity of this assay for busulfan in the presence of its potential metabolites was demonstrated. Also, plasma samples containing co-administered drugs gave no response under these conditions. Clinical samples obtained following administration of a 1 mg/kg oral busulfan dose demonstrate the applicability of this method to analysis of total and free plasma concentrations.

Introduction

Busulfan, a bifunctional alkylating agent, is frequently used in high-doses (16 mg/kg) and in combination with other agents as a preparative regimen for bone marrow transplantation procedures. Significant non-hematological toxicity, especially veno-occlusive disease of the liver [1, 5–7, 17, 20, 21, 23, 25, 29, 32], is observed with high-dose busulfan therapy. Toxicity reports demonstrating a relationship between the severity of toxicity and busulfan pharmacokinetic parameters [7, 15, 19, 24] have prompted investigations into individualized busulfan dosing based on monitoring first dose pharmacokinetics [9, 31].

Previously reported methods for busulfan analysis include high-performance liquid chromatography (HPLC) [2, 11, 12, 14, 23], gas chromatography-mass spectrometry (GC-MS) [4, 27] and GC with electron-capture detection (ECD) [3, 10, 11]. The HPLC methods described in the literature are not appropriate for routine analysis of busulfan due to the presence of interfering peaks, poor sensitivity [14, 18] or the requirement for a radioactive label [11, 12]. Both GC-MS of the sodium iodide derivative [4, 28, 30, 31], and GC-ECD of the sodium iodide [10, 13] and 2,3,5,6-tetrafluorothiophenol (TFTP) [3, 8] derivatives offer the sensitivity and reproducibility required for pharmacokinetic evaluation of busulfan. The GC-MS procedures have costly equipment requirements and the specificity of GC-ECD methods using sodium iodide derivatization is uncertain [10]. Although the measurement of non-protein bound (free) busulfan using HPLC with diethyldithiocarbamate derivatization has previously been reported [14], the clinical utility of this assay was not assessed.

This report describes the validation of a GC-ECD assay method using TFTP derivatization for quantitation of total and free busulfan plasma concentrations. 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 and therapeutic drug monitoring of busulfan in the clinical setting.

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Correspondence to: L. Embree, Medical Oncology Laboratory-Operations, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, British Columbia, Canada

Materials and methods

Gas chromatography. A model 5890 Hewlett Packard gas chromatograph (Hewlett Packard, 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 ID) with a film thickness of 0.20 μm (Supelco, Belleforte, Pa.). Helium (UPH, Linde, Medigas, Vancouver, B. C.) was used as carrier gas at a column flow rate of 1.5 ml/min with nitrogen gas (Linde) for ECD purge (6.0 ml/min) and makeup gas (55 ml/min). A split ratio of 1 : 20 was used with a column temperature program which increased from the initial temperature of 232° C to 250° C in 6 min. Injection volumes of 1 μl and 5 μl were made for plasma and plasma ultrafiltrate samples respectively.

Equipment. Synthesis and sample processing required the following: Silencer H-103N centrifuge (Western Scientific Services, Ltd., Richmond, B. C.); J21C refrigerated centrifuge (rotor JA 20.1) (Beckman Instruments, Inc., Palo Alto, Calif.); Vortex Genie (Fisher Scientific, Fairlawn, N. J.); Vortex-Evaporator heating block (Buchler Instruments, Fort Lee, N. J.); Labquake Shaker rotators (Labindustries, Inc., Berkeley, Calif.); Reacti-Vap drying apparatus (Pierce Chemical Co., Rockford, Ill.); Brinkmann Rotavapor (Buchi, Switzerland).

Materials. Busulfan, 1,6-hexanediol, sulfolane, tetrahydrothiophene 1-oxide, methanesulfonyl chloride, pyridine (HPLC grade), ethanol (spectroscopic grade) and TFTP were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Dichloromethane and methanol purchased from Fisher Scientific Co. (Fairlawn, N. J.) were HPLC grade. The following chemicals were also used: 3-sulfolene (Eastman Organic Chemicals, Rochester, N. Y.); potassium hydroxide and sodium hydroxide (Aristar grade, BDH Chemicals Ltd., Poole, England); sulfuric acid (Baker Chemical Co., Phillipsburg, N. J.); chloroform (Baker); anhydrous sodium sulfate (Mallinckrodt, Paris, Ky.); carboplatin (Bristol Laboratories, Belleville, Ont.); phenytoin (Smith & Nephew, Lachine, Que.); cyclophosphamide (Horner, Montreal, Que.) and cytarabine (Upjohn Co., Don Mills, Ont.). Filter paper was purchased from Fisher Scientific and the Centrifree Micropartition units (molecular mas cutoff 30 000 Da) for ultrafiltration of plasma were supplied by Amicon (Beverly, Mass.).

Synthesis of 3-hydroxysulfolane. 3-Hydroxysulfolane was synthesized according to Onkenhout et al. [22] with 48 h for reaction at room temperature and sulfuric acid neutralization. Final extraction with methanol (150 ml) followed by removal of residual water (with anhydrous sodium sulfate) and evaporation under reduced pressure left a viscous amber liquid. Yield was 12.3 g (85%). Purity was confirmed by a single peak on GC (with flame ionization detection) suggesting at least 99% purity. Nuclear magnetic resonance (NMR) ($CDCl_3$): δ2.1–2.5 (-CH_2, multiplet), 3.0–3.3 (-CH_2-S, multiplet), 3.55 (-OH, broad singlet), 4.6–4.8 (-CHOH, multiplet). Electron impact MS gave a base peak at m/e 44 and a molecular ion peak at m/e 136 with extensive fragmentation.

Synthesis of 1,6-bis(methanesulfonyloxy)hexane. 1,6-*Bis*(methanesulfonyloxy)hexane was synthesized from 1,6-hexanediol for use as internal standard by the procedure described for preparation of 1,8-*bis*(methanesulfonyloxy)octane [3]. White crystals were produced with a yield of 71%. A melting point of 56–57° C was observed (literature m. p. 60° C [16]). NMR ($CDCl_3$), δ1.4–1.5 (-OCH_2CH_2, multiplet), 1.7–1.8 (-CH_2, triplet), 3.0 (-CH_3, singlet), 4.2 (-CH_2O, triplet).

Synthesis of TFTP derivatives. The TFTP derivatives of busulfan (1,4-*bis*(2,3,5,6-tetrafluorophenylthio)butane) and the internal standard (1,6-*bis*(2,3,5,6-tetrafluorophenylthio)hexane) were prepared as described by Chen et al. [3] for busulfan. Purity for both derivatives was confirmed by a single peak on GC analysis (with ECD). The identity of these compounds was confirmed by NMR ($CDCl_3$) and electron impact MS.

Preparation of standard solutions 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 in ethyl acetate were made to the following final concentrations: 2.50, 5.00, 12.5, 25.0, 30.0, 42.5, 50.0 and 75.0 μg/ml for use as the total busulfan working solutions. Free busulfan working solutions were prepared by further diluting these solutions with ethyl acetate to provide concentrations from 0.500 to 10.0 μg/ml. Solutions of busulfan (1.50 and 0.300 μg/ml) were prepared for determination of assay sensitivity. Internal standard solutions of 40.0 and 8.00 μg/ml were prepared for quantitation of total and free busulfan respectively and for use as external standard in the extraction recovery studies. Busulfan and internal standard solutions were stored at room temperature and were stable for up to 3 months. Sodium hydroxide (1 M) in HPLC grade water was prepared on a monthly basis. The derivatization reagent solution was prepared just prior to use by mixing TFTP (0.45 ml) and methanol (2 ml). Calibration curve samples were prepared by adding 20 μl of the final busulfan working solutions to plasma (0.5 ml) and plasma ultrafiltrate (0.1 ml).

Extraction and derivatization. A modified extraction and derivatization procedure previously described [3] was used. The appropriate internal standard solution (20 μl) was added to plasma (0.5 ml) or plasma ultrafiltrate (0.1 ml) in 13 × 100 screw-capped PTFE-lined glass tubes. Following the addition of ethyl acetate (2 ml), all tubes were capped, vortex-mixed for 2 s and rotated for an additional 2 min. Samples were centrifuged for 10 min at 1000 *g*, the organic phase transferred to a clean 16 × 125 mm or 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 aqueous sodium hydroxide (20 μl) were added to all samples. Tubes were then capped, vortex-mixed for 2 s and heated at 70° C for 15 min. After derivatization, sodium hydroxide solution (2 ml) and hexane (2 ml) were added to all samples followed by vortex-mixing for 2 s, rotating for 2 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 for plasma samples and 5 μl for plasma ultrafiltrate samples).

Assay validation. Plasma and plasma ultrafiltrate calibration curves, each consisting of eight or seven spiked samples respectively, were prepared and assayed in quadruplicate on three occasions to evaluate linearity, precision and accuracy.

The recovery of busulfan from plasma and plasma ultrafiltrate samples and derivatization efficiency were evaluated. Busulfan recovery from plasma and plasma ultrafiltrate samples was determined by comparison of peak area ratios (busulfan/external standard) of extracted ($n = 9$) to unextracted ($n = 9$) samples at busulfan concentrations of 0.100, 1.00, 2.00 μg/ml for ultrafiltrate samples and 0.100, 1.00 and 3.00 μg/ml for plasma samples. Comparing the busulfan peak areas for the unextracted samples with peak areas obtained from samples containing equivalent amounts of the TFTP busulfan derivative provided the efficiency of the derivatization procedure. Plasma samples ($n = 6$) spiked with 1.00 μg/ml busulfan were extracted and derivatized at 70° C for 15, 30, 60 and 120 min to determine the effect of derivatization time on peak area.

Plasma and plasma ultrafiltrate samples containing 0.0600 μg/ml prepared in quadruplicate were extracted, derivatized and assayed to determine assay sensitivity.

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

Clinical samples. Three patients undergoing treatment with a busulfan-containing preparative regimen (1 mg/kg every 6 h for 16 doses) gave informed consent to this study which was approved by the University of British Columbia Ethics Committee and the local hospital ethics review boards. Blood samples were collected in EDTA Vacutainer tubes (Becton Dickinson Canada Inc., Mississauga, Ont.) just prior to and 15, 31, 60, 120 and 360 min after administration of the first busulfan dose. Samples were kept at 4° C, centrifuged within 2.5 h and the plasma was withdrawn. Plasma ultrafiltrate, prepared by centrifuging approximately 1 ml of plasma in a Centrifree Micropartition unit for 30 min at 2000 *g*, frozen (–20° C) along with the plasma until analysis. Plasma and plasma ultrafiltrate samples were analysed in duplicate by the GC-ECD assay procedures above.

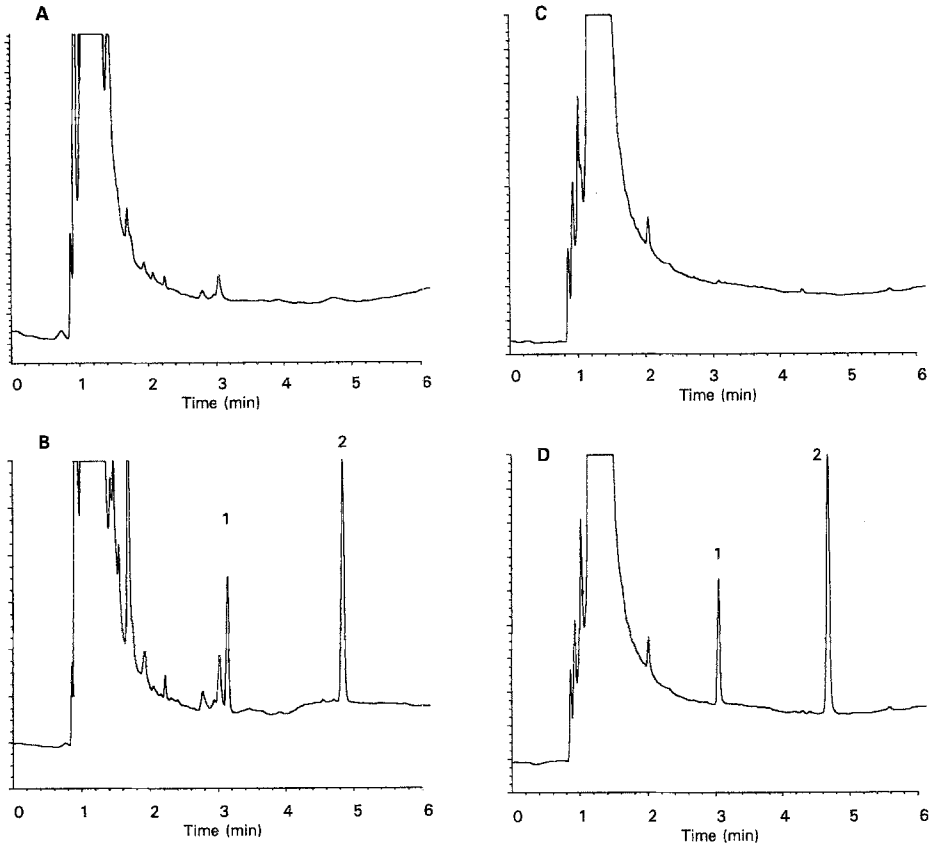

Fig. 1. Representative chromatograms of extracted and derivatized **A** blank plasma, **B** 0.500 μg/ml spiked plasma sample, **C** blank plasma ultrafiltrate and **D** 0.500 μg/ml spiked plasma ultrafiltrate sample. Peaks: 1 = busulfan TFTP derivative; 2 = internal standard derivative

Results

Recovery

Ethyl acetate extraction efficiency for both plasma and plasma ultrafiltrate assays was determined by comparison of the peak area ratios (busulfan/external standard) for extracted and unextracted samples at concentrations from 0.100 to 3.00 μg/ml. Extraction efficiencies ranged from 78.4% to 85.8% for total busulfan and 85.9% to 89.6% for free busulfan but did not increase directly with concentration. Derivatization yield for plasma and plasma ultrafiltrate samples, determined by comparison of peak areas from the unextracted samples with those from direct injection of equivalent amounts of the TFTP busulfan derivative, ranged from 56.0% to 71.3% (mean 63.7%). Plasma samples spiked with busulfan and derivatized for 15, 30, 60 and 120 in at 70°C ($n = 6$ at each time point) gave mean (±SD) busulfan peak areas of 14112 (±1360), 14369 (±1580), 14806 (±1620) and 14685 (±860), respectively.

Specificity, precision and sensitivity

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, carboplatin, ondansetron, cytarabine and cyclophosphamide. Chromatograms obtained from analysis of plasma and plasma ultrafiltrate are shown in Fig. 1.

Precision data for the total and free procedures, determined from the calibration curve validation experiment, are listed in Table 1. The maximum sensitivity obtained for both the plasma and plasma ultrafiltrate procedures was at a concentration of 0.0600 μg/ml with a signal-to-noise ratio of 5.

Calibration curves

Regression analysis of the calibration curve validation data demonstrated that Eq. 1, relating area ratio and busulfan

Table 1. Precision data for GC-ECD assay of busulfan in plasma and plasma ultrafiltrate

Concentration (μg/ml)	Plasma peak area ratio (busulfan/internal standard) (mean ±SD; % CV)	Plasma ultrafiltrate peak area ratio (busulfan/internal standard) (mean ±SD; % CV)
0.100	0.0916±0.00576; 6.29	0.0853±0.00782; 9.17
0.200	0.178 ±0.0176; 9.89	0.168 ±0.0110; 6.55
0.500	0.405 ±0.0377; 9.31	0.405 ±0.0234; 5.80
1.00	0.777 ±0.0468; 6.02	0.819 ±0.0701; 8.56
1.20	0.967 ±0.0633; 6.55	1.03 ±0.0979; 9.50
1.70	1.36 ±0.0762; 5.60	1.37 ±0.0872; 6.36
2.00	1.60 ±0.0675; 4.22	1.65 ±0.135; 8.18
3.00	3.11 ±0.100; 3.22	

Table 2. Statistics for predicted total busulfan concentrations in plasma

Actual concentration (μg/ml)	Mean	Bias	Standard deviation	% Coefficient of variation
0.100	0.123	0.023	0.0190	15.5
0.200	0.234	0.034	0.0276	11.8
0.500	0.517	0.017	0.0431	8.34
1.00	0.977	–0.003	0.0533	5.46
1.20	1.21	0.01	0.0760	6.28
1.70	1.70	0	0.0970	5.71
2.00	2.00	0	0.0915	4.58
3.00	3.04	0.04	0.300	9.87

Table 3. Statistics for predicted free busulfan concentrations in plasma ultrafiltrate

Actual concentration (μg/ml)	Mean	Bias	Standard deviation	% Coefficient of variation
0.100	0.103	0.003	0.0195	18.9
0.200	0.204	0.004	0.0221	10.8
0.500	0.496	–0.004	0.0351	7.08
1.00	1.00	0	0.104	10.4
1.20	1.25	0.05	0.0942	7.54
1.70	1.67	–0.03	0.116	6.95
2.00	2.02	0.02	0.172	8.51

concentration, described the calibration curves for both total and free busulfan. An overall correlation coefficient of 0.99 was obtained for 12 curves from each assay method.

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

To examine the ability of the calibration curve to predict concentration values, the calibration curve validation data (for total and free busulfan determination) was divided into four calibration curves for each day. Predicted concentration values for three curves 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 12 values per concentration). The statistical summary of the predicted values is given in Tables 2 and 3 for the total and free busulfan assay methods. One-way analysis of variance indicated that there was no significant difference in the predicted levels on the three different days for either assay. Good agreement between the actual and predicted concentrations demonstrated the accuracy of these assay procedures.

Clinical samples

Plasma and plasma ultrafiltrate samples from three patients were evaluated with the assay procedures described in this report. Representative chromatograms obtained on analysis of the pre-treatment (patient blank) and 60 min samples from one patient are shown in Fig. 2. The range of total and free busulfan concentrations was found to be 0.100–1.50 μg/ml and 0.100–1.0 μg/ml, respectively.

Discussion

Our modifications to the previously published GC-ECD assay [3] primarily involved development and validation of a sample handling procedure for determination of free busulfan at therapeutically relevant concentrations, reduction of sample handling time and the use of a fused silica capillary column for the chromatographic separations and 1,6-*bis*(methanesulfonyloxy)hexane as internal standard. The added efficiency of the capillary column and the use of a shorter chain internal standard has allowed resolution of busulfan from endogenous compounds with a run time of 6 min. Chromatograms of blank and busulfan spiked (0.500 μg/ml) plasma and plasma ultrafiltrate samples shown in Fig. 1 contain peaks from endogenous compounds eluting prior to busulfan and no interference with either busulfan or the internal standard. Similarly, the chromatograms in Fig. 2, obtained from a patient receiving busulfan therapy at a dose of 1 mg/kg, demonstrate that there are no interfering peaks in the pre-treatment sample for either total or free assay methods.

Recovery of busulfan from plasma observed here is similar to results previously reported [3]. The derivatization yield using the present method was approximately 28% lower [3], which could not be attributed to the decrease in reaction time at 70° C. Using the 15 min derivatization time, reproducible results were obtained as demonstrated by the area ratio precision data and mean percentage coefficient of variation values of 6.39% and 7.73% for the total and free assay methods, respectively.

Calibration curve analysis demonstrates assay linearity over the concentration range evaluated with correlation coefficients of 0.99 for both total and free busulfan determinations with no day-to-day variability. A limit of detec-

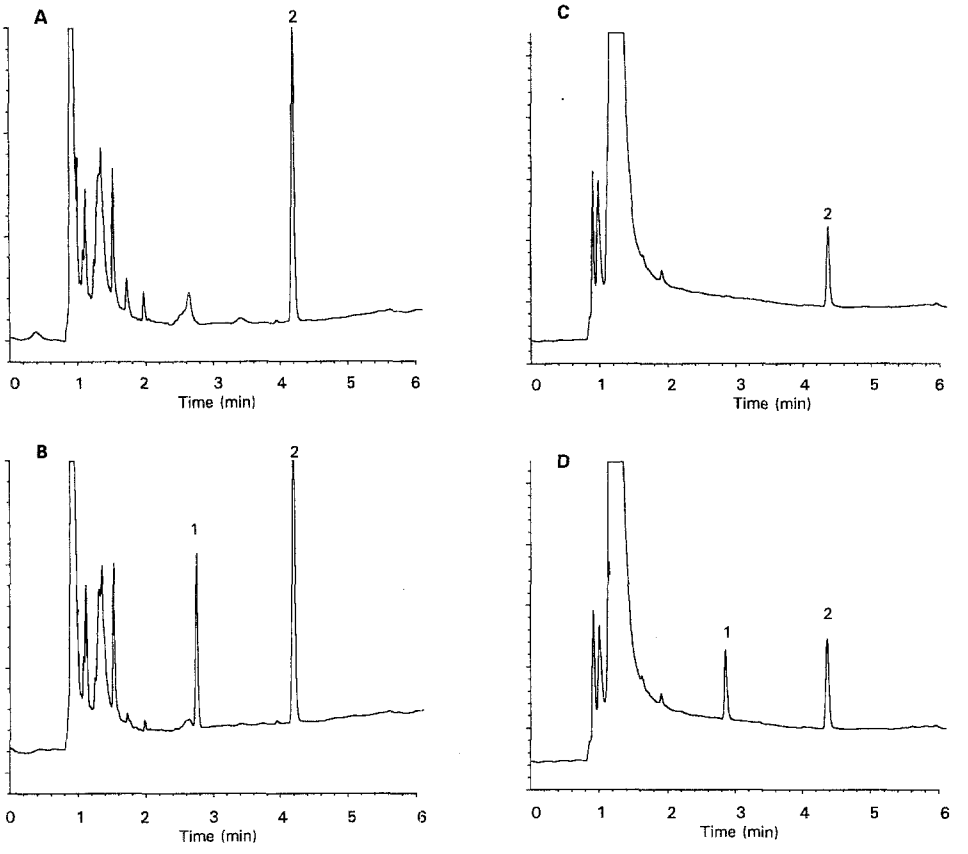

Fig. 2. Chromatograms of extracted and derivatized **A** patient pre-treatment plasma, **B** patient plasma sample 1 h post-administration of an oral dose of busulfan (1 mg/kg), **C** patient pre-treatment plasma ultrafiltrate and **D** corresponding patient plasma ultrafiltrate sample taken 1 h post-administration. Peaks: 1 = busulfan TFTP derivative; 2 = internal standard derivative

tion of 0.0600 μg/ml (with a signal-to-noise ratio of 5) and limit of quantitation of 0.100 μg/ml plasma or plasma ultrafiltrate were observed and are similar to previously reported values for plasma samples [3].

The assay methods for GC determination of total and free busulfan concentrations meet the requirements for pharmacokinetic studies [26] in addition to the time constraints on assay methods to be used for therapeutic monitoring. Clearly, there is more opportunity for individualized doses of busulfan to be beneficial when administered early in therapy. As demonstrated by the plasma and plasma ultrafiltrate data obtained to date from our patients and those described by Chen et al. [3], the assay methods described here offer sufficient sensitivity and specificity to be able to quantitate total and free busulfan concentrations in clinically relevant samples. The assay methods described herein will be used to evaluate total and free busulfan pharmacokinetic and pharmacodynamic behavior when administered as part of bone marrow transplantation preparative regimens.

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