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DETERMINATION OF BUSULFAN IN HUMAN PLASMA BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

TIAN-LING CHEN, LOUISE B. GROCHOW*, LISA A. HUROWITZ and ROBERT B. BRUNDRETT

The Johns Hopkins Oncology Center, The Johns Hopkins University, School of Medicine, 600 North Wolfe Street, Baltimore, MD 21205 (U.S.A.)

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SUMMARY

A simple and highly sensitive gas chromatographic method has been developed for the determination of busulfan in human plasma. After extraction of plasma specimens (clinical or spiked) with ethyl acetate, busulfan and the internal standard [1,8-bis(methanesulfonyloxy)octane] were derivatized with 2,3,5,6-tetrafluorothiophenol to yield compounds monitored by a ^{63}Ni electron-capture detector. Sample recoveries from extraction and derivatization were greater than 78 and 91%, respectively. The limit of quantitation was 0.01 μg/ml (0.04 μM) in 1 ml of plasma with a linear relationship over the 0.01–1.0 μg/ml (0.04–4 μM) concentration range. The method has been applied to analyze the plasma versus time profile of busulfan in human subjects following administration of an oral dose of 4 mg/kg per day as a marrow ablative chemotherapy for bone marrow transplantation.

INTRODUCTION

Busulfan [Myleran, 1,4-bis(methanesulfonyloxy)butane, I, Fig. 1] is a straight-chain bifunctional alkylating agent which has been used primarily in chronic myelocytic leukemia (CML) and polycythemia rubra vera at low daily oral doses over the past 30 years [1]. Since the late 1970s, high-dose busulfan (1 mg/kg every 6 h for 48–96 h) with cyclophosphamide has been employed as a preparative regimen for bone marrow transplantation, and experience with this regimen has produced long-term remission in both adults and children with malignancies [1–4]. However, the available information about its pharmacokinetic behavior, especially at this high-dose range in humans, has been limited by the lack of simple and sensitive analytical methods. Severe toxicities such as hepatic veno-occlusive disease and diffuse interstitial pneumonitis were seen in intensive chemotherapy with busulfan and other cytotoxic agents [1,4]. It is possible that these toxicities are related to variations in absorption and disposition of the drug.

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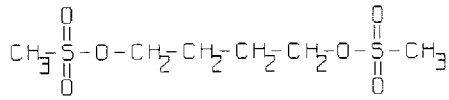
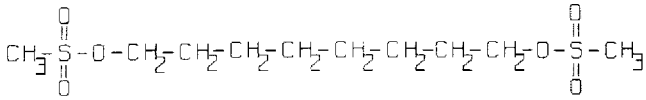
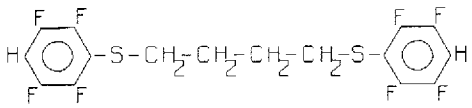


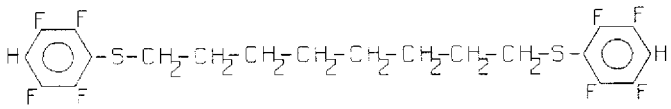
I. BUSULFAN



II. INTERNAL STANDARD



III. BUSULFAN DERIVATIVE



IV. INTERNAL STANDARD DERIVATIVE

Fig. 1. Chemical structures of busulfan, internal standard and their derivatives.

Previously published busulfan assays involving gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring and high-performance liquid chromatography (HPLC) with UV detection have been restricted by either the costly equipment required or insufficient sensitivity [8,9]. To overcome these limitations, a new GC procedure for the assay of derivatized busulfan in human plasma has been developed using electron-capture detection (ECD). It should be useful for pharmacokinetic and bioavailability studies of busulfan in humans.

EXPERIMENTAL

Instrumentation and conditions

A Model 3400 gas chromatograph (Varian, Sugar Land, TX, U.S.A.) equipped with a ^{63}Ni (8 mCi) electron-capture detector was employed. Separations were performed on a 1.82 m × 2 mm I.D. glass coil packed with 3% SP2250 on 80–100 mesh Supercoport (Supelco, Bellefonte, PA, U.S.A.). Nitrogen was used as carrier gas. The column was conditioned with a nitrogen flow-rate of 20 ml/min at 280°C for 24 h. The operating conditions were as follows: the injector and detector temperatures were 250 and 300°C, respectively. Nitrogen flow-rate was 27.5 ml/min. A two-stage column temperature program was used: the initial temperature was 230°C, then the column was heated at 4°C/min to 250°C for 5 min, this was followed by an increase at 12°C/min to 274°C in order to remove contaminations from the column. The sensitivity of the detector was fixed at 256 × 10 (attenuation × range) for calibration curves and patient samples, which gave a

good response with adequate background noise. Chromatograms were traced on a Model 1200 recorder (Linear, Reno, NV, U.S.A.) at 1 cm/min.

Reagents

All chemicals except organic solvents were obtained from Aldrich (Milwaukee, WI, U.S.A.). Methanol and ethyl acetate were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

1,8-Bis(methanesulfonyloxy)octane (II, Fig. 1) was used as an internal standard and was synthesized as follows: a solution of methanesulfonyl chloride (25 g, 0.22 mol) in methylene chloride (40 ml) was added slowly to a solution of 1,8-octanediol (14.6 g, 0.1 mol), pyridine (17.4 g, 0.22 mol) and methylene chloride (40 ml) while stirring at 0°C. The mixture was stirred at 25°C for 1 h, extracted three times with water and evaporated at reduced pressure. Crystallization from ethanol yielded 26.8 g (90%) while crystals, m.p. 64–65°C (Lit. m.p. 67.5°C) [10].

Crystalline 1,4-bis(2,3,5,6-tetrafluorophenylthio)butane (busulfan derivative, III, Fig. 1) for use in peak identification was synthesized as follows: a mixture of 2,3,5,6-tetrafluorothiophenol (TFTP) (540 mg, 3 mmol), busulfan (370 mg, 1.5 mmol), aqueous sodium hydroxide (3 mmol of 1 *M*) and methanol (3 ml) was refluxed for 2 h. Water and methylene chloride were added and the organic phase was separated and evaporated at reduced pressure. Crystallization from methanol gave 400 mg (65%) white crystals, m.p. 60–62°C. NMR (C^2HCl_3), δ 7.0 (2H, t of t), 2.9 (4H, m), 1.7 (4H, m).

Stock solutions

An appropriate quantity of busulfan and internal standard was accurately weighed and dissolved separately in 10 ml of ethyl acetate to prepare 2.5 and 8.2 mg/ml solutions, respectively. Sequential dilutions to 2.5 and 8.2 μg/ml were then made as working solutions. These solutions were stored at room temperature and were stable for at least three months. The other reagent solutions, 1.5 *M* TFTP in methanol and 1 *M* sodium hydroxide solution in water, were freshly made prior to use.

Extraction and derivatization of plasma samples

Plasma (1 ml) and 8.2 μg/ml internal standard solution (20 μl) were pipetted into 16×125 mm screw-capped glass tubes. This was followed by 3 ml of ethyl acetate. The tubes were capped and vigorously shaken by hand for 2 min. After centrifugation for 10 min at 1000 *g*, the organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.2 ml water and 20 μl of 1.5 *M* TFTP in methanol and 20 μl of 1 *M* sodium hydroxide were added. The solution was heated at 70°C in a heating block for 2 h. An additional 5 ml of 1 *M* sodium hydroxide was added, and the reaction products were extracted with 5 ml of ethyl acetate. The mixture was again agitated and separated by centrifugation (1000 *g* for 10 min). A 2-μl aliquot was injected into the gas chromatograph.

Calibration curves were produced by adding 0–40 μl of 2.5 μg/ml busulfan working solution to drug-free plasma samples prior to adding internal standard.

Quantitation and statistical analysis

The calibration curve was constructed by plotting peak-height ratios of busulfan to internal standard as a function of concentration of busulfan over the range of 0.1–1.0 μg/ml (0.4–4 μ*M*). The best-fit line was determined by least-squares linear regression. Plasma concentrations of busulfan in unknown samples were calculated by interpolation from the calibration curve.

Precision parameters of the assay have been investigated within-run as well as between-run on five days over a two-week period. Two concentrations of spiked plasma samples, 0.5 μg/ml (2 μ*M*) and 1.0 μg/ml (4 μ*M*), were used for both experiments. Coefficient of variation was calculated from deviation of the arithmetic mean value of peak-height ratios at each concentration.

The recovery studies included both extraction efficiency and reaction (derivatization) efficiency. The extraction efficiency of busulfan from spiked human plasma was determined by comparing the peak heights of extracted plasma samples to those of unextracted samples (busulfan added directly to derivatization reagents). The reaction efficiency of busulfan was determined by comparing the peak height of unextracted samples to those of prepared reference samples containing the equivalent amounts of crystalline busulfan derivative. At each of the three concentrations used duplicate samples were measured.

Linear response of detector was tested by making duplicate injections of a series of plasma standards ranging from 0.01 to 10 μg/ml.

RESULTS AND DISCUSSION

Representative chromatograms obtained from plasma analysis and pure crystalline derivative are depicted in Fig. 2. Retention times of busulfan and internal standard derivatives (III and IV, Fig. 1) were 3.0 and 7.5 min, respectively. Both peaks were symmetrical with good baseline resolution and minimum tailing. There were no interfering peaks in plasma from 30 patients at the retention times corresponding to busulfan and internal standard derivatives.

Calibration curves were highly linear with negligible y-intercepts and yielded correlation coefficients of 0.99 or greater. Precision data for within-run and between-run are summarized in Table I.

Recovery studies were completed at three concentrations of spiked plasma samples: 0.1 μg/ml (0.4 μ*M*), 0.5 μg/ml (2 μ*M*) and 1.0 μg/ml (4 μ*M*). Extraction efficiencies of busulfan from plasma into ethyl acetate were 78, 79 and 83%, respectively. Reaction efficiencies of busulfan were 92, 93 and 91%, respectively. The results indicated that varying the concentrations of busulfan in plasma between 0.1 and 1.0 μg/ml (0.4–4 μ*M*) had no noticeable effect on recovery.

The detector response was linear over the range of 0.01–1.0 μg/ml (0.04–4 μ*M*) with linear regression equation of $y = 1.03\,x + 0.05$ and correlation coefficient of 0.9974 (Fig. 3). Concentrations of busulfan greater than 1.0 μg/ml (4 μ*M*) sat-

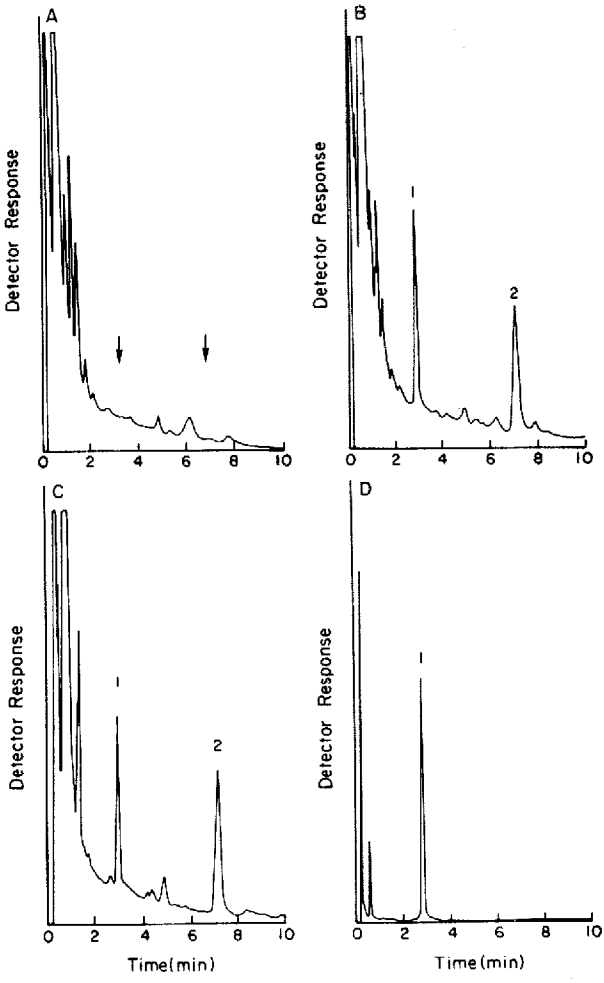


Fig. 2. Chromatograms of (A) derivatized blank human plasma, (B) 1 μg/ml spiked plasma sample, (C) patient plasma sample 75 min after an oral dose of 1 mg/kg and (D) pure crystalline busulfan derivative. Peaks: 1 = busulfan (derivative); 2 = internal standard (derivative).

TABLE I

PRECISION OF GC–ECD ASSAY FOR BUSULFAN

Concentration* (μg/ml)	Busulfan/internal standard ratio (mean ± S.D.)	Coefficient of variation (%)
Intra-day (n = 10)		
0.5	0.59 ± 0.03	5.08
1.0	1.03 ± 0.07	6.80
Inter-day (n = 5)		
0.5	0.75 ± 0.05	6.67
1.0	1.52 ± 0.09	5.92

*A 1-ml plasma sample was spiked at the indicated concentration.

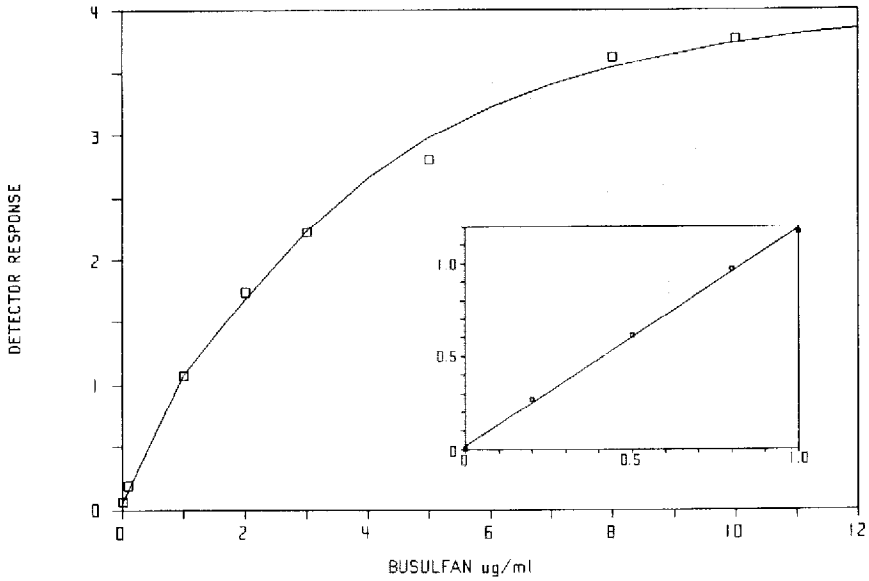


Fig. 3. Linearity of GC–ECD assay for busulfan.

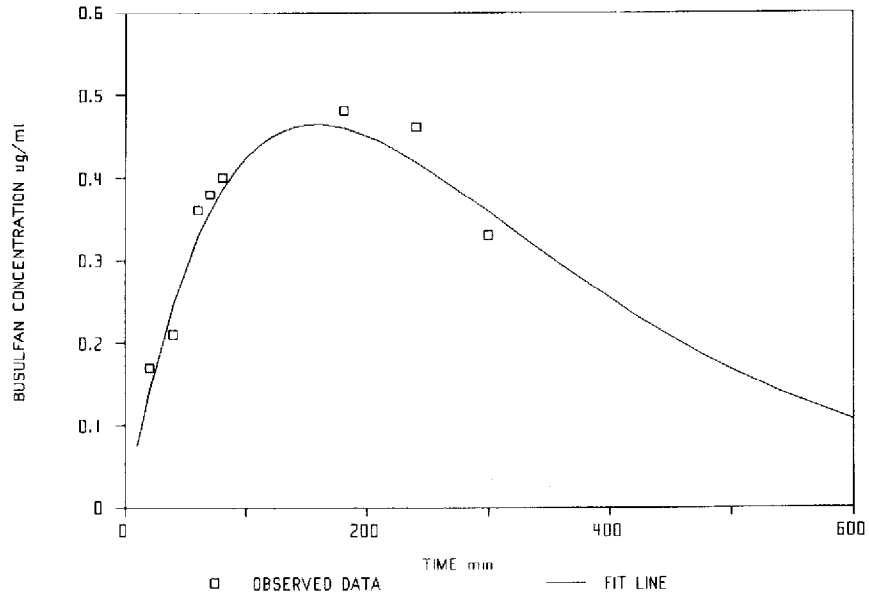


Fig. 4. Representative plasma concentration–time profile of busulfan following an oral dose of 1 mg/kg in a patient.

urated the detector system and had to be diluted to fit into the concentration range of calibration curve before injection.

The sensitivity of the assay, defined as the minimum drug concentrations corresponding to two times signal-to-noise ratio, was found to be 0.01 μg/ml (0.04

μM) in 1 ml of plasma sample at 128×10 (attenuation×range). Higher sensitivity of the assay may be obtained by the use of a smaller dilution volume during sample extraction after derivatization. This should be sufficiently sensitive for studies of busulfan kinetics in human when administered at conventional doses of 2–6 mg per day.

To date the described method has been successfully used to analyze over 500 samples obtained 0–6 h after administration of a 1 mg/kg oral dose of busulfan to human subjects in an ongoing pharmacokinetic study. The range of busulfan concentration was found to be 0.1–1.3 μg/ml (0.4–5 μM), indicating that the calibration range (0.1–1.0 μg/ml or 0.4–4 μM) for the assay was adequate. Fig. 4 shows a typical busulfan plasma concentration–time profile in a human subject after an oral dose of 1 mg/kg.

In conclusion, the new GC method described in this study is simple, sensitive, and permits use of small sample sizes appropriate for pediatric as well as adult populations. It should be suitable in most laboratories with the available equipment, and sufficiently useful for further pharmacokinetic studies of busulfan at low and high dose in humans.

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