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Note

Gas chromatographic determination of busulfan in plasma with electron-capture detection

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Busulfan, an alkytating agent of the bis-(methanesulfonate) ester type, is frequently used in the treatment of chronic myelogenous leukemia. Recently a gas chromatographic (GC) procedure for the determination of busulfan in plasma was presented for the first time [1], which comprised an initial extraction of plasma with dichloromethane followed by evaporation and derivatization of busulfan using sodium iodide in acetone. The formed derivative was purified by solvent extraction. The GC step utilized a packed column in conjunction with selected ion monitoring (SIM).

In the present paper a new derivatization technique is given, comprising derivatization and extraction of busulfan from plasma in a single step. High separation efficiency and sensitivity are obtained by the use of a capillary column in combination with electron-capture detection (ECD).

EXPERIMENTAL

Gas chromatography

The GC analysis was performed using a Varian 3700 instrument, equipped with a constant-current ^{63}Ni electron-capture detector. An OV-1 fused-silica capillary column (25 m × 0.3 mm I.D.) with a film thickness of 0.52 μm (Hewlett-Packard) was used. The original injector was substituted by an injection splitter (Model A, Cat. No. 8045) obtained from Chrompack, Middelburg, The Netherlands.

The original flow regulator was replaced by a Brooks 8601 pressure regulator (0—2.5 bar). The instrument was operated isothermally with the oven, detector and injector port temperatures at 145°C, 250°C and 200°C, respectively. The carrier gas (helium) flow-rate was 2 ml/min, with an inlet pressure of 0.4 bar.

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Make up gas (nitrogen), 30 ml/min, was added through the hydrogen inlet in the detector base. A split ratio of 1:10 was used unless otherwise stated.

A Varian 1400 instrument equipped with a flame ionization detector and a 1.5 m × 2 mm I.D. column packed with 10% SP 2401 on Supelcoport 100—120 mesh was used. The instrument was operated isothermally with oven, detector and injector port temperatures of 130°C, 260°C and 200°C, respectively. The carrier gas (nitrogen) flow-rate was 30 ml/min.

The GC—mass spectrometric analysis was carried out as described in ref. 1 using an LKB 2091 instrument. The liquid chromatographic (LC) conditions are described in ref. 1.

Reagents and chemicals

Busulfan was obtained from EGA-Chemie (Steinheim, F.R.G.) and 1,5-bis(methanesulfonoxy)pentane was prepared according to the method reported previously [1]. Sodium iodide and *n*-pentadecane were obtained from Merck (Darmstadt, F.R.G.). All solvents used were of analytical grade.

Reaction conditions

Busulfan was dissolved in acetone (0.1 ml) and mixed with phosphate buffer pH 7.0, ionic strength (I) = 0.1 to give a final busulfon concentration of 1.0 mg/ml. The aqueous phase was mixed with sodium iodide and *n*-heptane containing the internal standard (*n*-pentadecane 0.3 mg/ml). The reaction was carried out at 70°C under stirring with a micro-magnet in the tube. The sodium iodide concentration was varied within the range 1—4 M, having a constant phase ratio V_{org}/V_{aq} = 1. At appropriate times samples (0.1 ml) were withdrawn from the organic phase for analysis.

The influence of the ratio V_{org}/V_{aq} on the reaction rate was studied as above using 4 M sodium iodide and phase ratios V_{org}/V_{aq} within the range 0.2—5. The analysis was performed using GC—flame ionization detection (FID).

Determination of partition coefficients

The partition coefficients for busulfan and 1,4-diiodobutane were determined as described in ref. 1. The distribution of 1-methanesulfonoxy-4-iodobutane was studied using equal volumes of phosphate buffer pH 7.0 (I = 0.1) and *n*-heptane (equilibrium time 30 min at 25.0 ± 0.1°C). The initial concentration of 1-methanesulfonoxy-4-iodobutane in the aqueous phase and the concentration in the aqueous phase at the equilibrium stage were determined by LC.

Determination of busulfan in plasma

Plasma (1.00 ml) was mixed with 1,5-bis(methanesulfonoxy)pentane (0.10 ml, 1.0 μg/ml) in acetone and sodium iodide in water (1.0 ml, 8 M). After addition of *n*-heptane (0.40 ml), the reaction was carried out at 70°C for 40 min under stirring. Part of the organic phase (1—2 μl) was injected into the GC—ECD system using a split ratio of 1:10.

RESULTS AND DISCUSSION

Conversion of busulfan to 1,4-diiodobutane in aqueous solution

The reaction of busulfan with sodium iodide in acetone has been described recently [1]. In the present paper the reaction with sodium iodide was studied in aqueous solution with the ultimate goal of performing the derivatization directly in plasma to obtain a simplified work-up procedure preceding the GC step. The reaction of busulfan in aqueous solution using 4 *M* sodium iodide at 70°C gave a yield of 50% of 1,4-diiodobutane in about 15 min.

However, it was not possible to obtain a quantitative conversion to 1,4-diiodobutane, probably due to subsequent hydrolysis of the derivative formed. By performing the reaction in the presence of an organic solvent, the stability of 1,4-diiodobutane could be increased considerably. For example, 1,4-diiodobutane was stable for at least 50 h at 70°C in a two-phase system comprising equal volumes of phosphate buffer pH 7.0 and *n*-heptane, which must be attributed to the high partition of 1,4-diiodobutane to the organic phase ($K_d > 100$).

A quantitative yield of 1,4-diiodobutane was obtained in a two-phase system after about 30 min using 4 *M* sodium iodide (Fig. 1). The reaction was slower using 1 *M* and 2 *M* sodium iodide and the final yields were lower (75.5% and

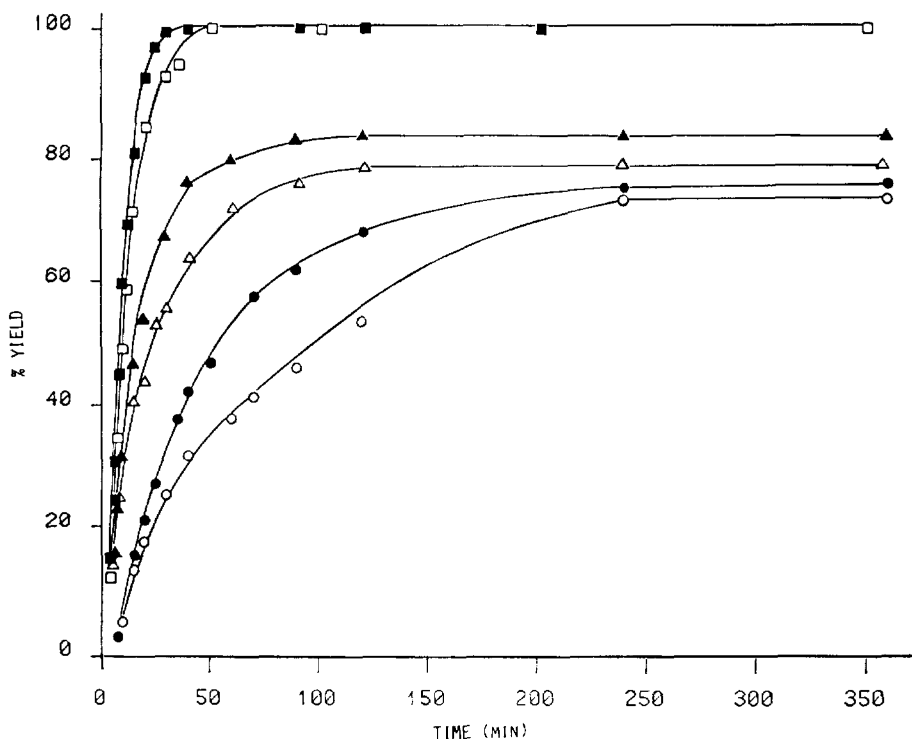

Fig. 1. Influence of sodium iodide concentration on the formation of 1,4-diiodobutane and 1,5-diiodopentane. Temperature: 70°C. The yields were calculated using 1,4-diiodobutane and 1,5-diiodopentane as reference. Key: sodium iodide 1 *M* (●), 2 *M* (▲), 4 *M* (■) with 1,4-diiodobutane as reference; sodium iodide 1 *M* (○), 2 *M* (△), 4 *M* (□) with 1,5-diiodopentane as reference.

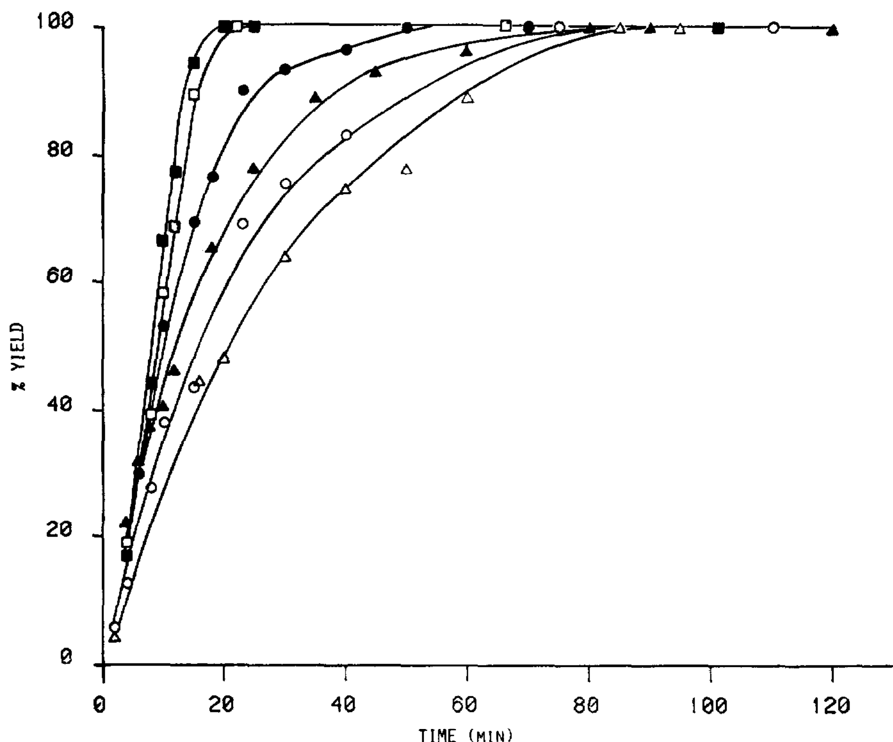

Fig. 2. Influence of ratio V_{org}/V_{aq} on the formation of 1,4-diiodobutane and 1,5-diiodopentane. Temperature: 70°C. The yields were calculated using 1,4-diiodobutane and 1,5-diiodopentane as reference. Key: V_{org}/V_{aq} ratio of 0.2 (▪), 1 (•), 5 (▴) with 1,4-diiodobutane as reference; V_{org}/V_{aq} ratio of 0.2 (□), 1 (○), 5 (△) with 1,5-diiodopentane as reference.

84.2%, respectively). A high concentration of iodide is obviously required to obtain a rapid and complete conversion of busulfan to 1,4-diiodobutane.

The reaction of busulfan with iodide takes place in the aqueous phase. Consequently, the overall reaction rate in the two-phase system should be affected by the fraction of busulfan and the intermediate (1-methanesulfonoxy-4-iodobutane) present in the aqueous phase.

The influence of the ratio V_{org}/V_{aq} on the rate of formation of 1,4-diiodobutane is illustrated in Fig. 2. An increased reaction rate is observed when the ratio is decreased. The major portion of busulfan is partitioned to the aqueous phase ($K_d < 0.01$) and hence a change of the ratio V_{org}/V_{aq} in the range studied (0.2—5) should only slightly affect the rate of formation of 1-methanesulfonoxy-4-iodobutane. However, 1-methanesulfonoxy-4-iodobutane is considerably more lipophilic than busulfan ($K_d = 2.0$) and a decreased ratio V_{org}/V_{aq} should increase its rate of conversion to 1,4-diiodobutane. For example, a change of the ratio from 5 to 0.2 will change the fraction of 1-methanesulfonoxy-4-iodobutane present in the aqueous phase from 30% to 90%.

The internal standard 1,5-bis(methanesulfonoxy)pentane has a lower reaction rate than busulfan. This is probably due to its more lipophilic character which will give a higher partition to the organic phase (Figs. 1 and 2).

Reaction of busulfan in plasma

The reaction of busulfan and the internal standard was carried out in a two-phase system using plasma containing 4 *M* sodium iodide as an aqueous phase and *n*-heptane as an organic phase at 70°C for 40 min. The yields of 1,4-diiodobutane and 1,5-diiodopentane were 91.0 ± 3.2% (C.V., $n = 5$) and 92.0 ± 3.5% (C.V., $n = 5$), respectively. A ratio V_{org}/V_{aq} of 0.2 is used in the general procedure for the determination of busulfan in plasma to give a rapid formation of 1,4-diiodobutane. Furthermore, a ratio V_{org}/V_{aq} of 0.2 gives a higher concentration of 1,4-diiodobutane in the organic phase as compared to the initial plasma concentration of busulfan, making further concentration steps unnecessary prior to the GC analysis.

Injection technique

Splitless injection [2, 3] is one of the most used techniques in quantitative trace analysis by capillary GC [4]. Generally the technique is performed isothermally using a high-boiling alkane as a solvent, when combined with ECD [5—7]. The quantitation of busulfan using the splitless technique with *n*-decane as the solvent resulted in a number of interfering peaks in the chromatograms even when the solvent was redistilled. However, no interfering peaks were found when *n*-heptane was used as a solvent in combination with a split injection technique.

A poor reproducibility was obtained when the peak heights from a number of identical injections were compared using split of ratios 1:2—1:40 (Table I). However, very good precision was obtained in the peak height ratios 1,4-diiodobutane/1,5-diiodopentane (Table I). The column efficiency was constant (H = 0.77 ± 0.04 mm, S.D.) using split ratios in the range 1:5—1:40 but it decreased by about 50% using a split ratio of 1:2.

TABLE I

EFFECT OF SPLIT RATIO ON PRECISION

Split	1,4-Diiodobutane peak height (mm) ± S.D.	1,5-Diiodopentane peak height (mm) ± S.D.	Peak height ratio ± S.D.
1:2	396 ± 63	463 ± 73	0.85 ± 0.01
1:5	227 ± 26	248 ± 28	0.91 ± 0.01
1:10	126 ± 11	137 ± 12	0.92 ± 0.01
1:15	90 ± 11	98 ± 12	0.92 ± 0.01
1:25	70 ± 5	76 ± 5	0.91 ± 0.01
1:40	42 ± 7	46 ± 8	0.92 ± 0.01

Detection, selectivity and precision

The minimum detectable concentration [8] obtained by ECD is given in Table II. The value for 1,4-diiodobutane corresponds to a minimum detectable quantity of 30 fg (retention time 8 min). The sensitivity of the EC detector in combination with the high separation efficiency of the capillary column, makes the determination of busulfan in patient plasma possible in the low ng/ml level without any interfering peaks (Fig. 3). The standard curve using plasma was linear within the range studied, 5—300 ng/ml. A least-squares

TABLE II

MINIMUM DETECTABLE CONCENTRATION (MDC) OF 1,4-DIIODOBUTANE AND 1,5-DIIODOPENTANE

Compound	MDC* $\times 10^{16}$ mol/sec
1,4-Diiodobutane	0.44
1,5-Diiodopentane	0.54

*Signal-to-noise ratio = 3.

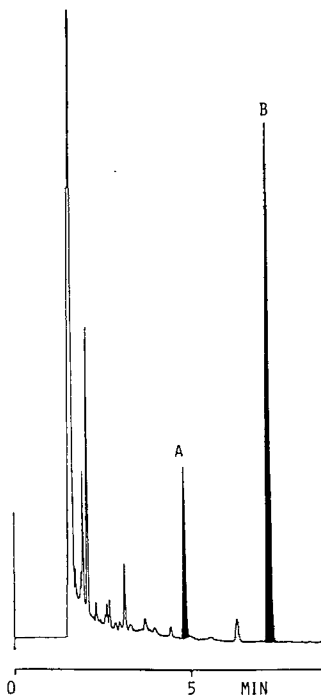

Fig. 3. Chromatogram obtained from patient plasma containing 14 ng/ml busulfan. Split: 1:10. Key: A = busulfan as 1,4-diiodobutane; B = 1,5-bis(methanesulfonoxy)pentane as 1,5-diiodopentane.

analysis gave a slope of $1.64 \times 10^{-2} \pm 0.04 \times 10^{-2}$ (S.E.M.), an intercept of $9.70 \times 10^{-2} \pm 5.50 \times 10^{-2}$ (S.E.M.) and a correlation coefficient of 0.9988.

The precision of the analytical method was ± 3.9% (C.V.) at the 10 ng/ml level ($n = 5$) and ± 2.3% (C.V.) at the 100 ng/ml level ($n = 5$).

Evaluation of the method

The prechromatographic procedure is more rapid and labour saving than in the GC—SIM method [1], and the technique requires less-expensive instrumentation.

The accuracy of the method was corroborated by analysis of patient plasma using GC—SIM [1]. The results obtained by GC—ECD were in good agreement

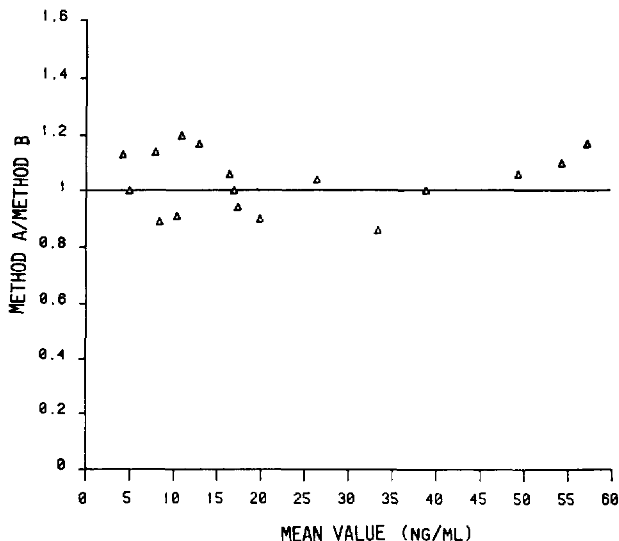

Fig. 4. Method-comparison analysis. Method A: data from analysis by GC—ECD. Method B: data from analysis by GC—SIM.

with those obtained by GC—SIM, when plotted according to ref. 9 (Fig. 4). The quotient of ECD values/SIM values was 1.043 ± 0.111 (S.D.). It should be pointed out, however, that the metabolic pattern of busulfan is poorly characterized, and since metabolites of busulfan might be converted to 1,4-diiodobutane by performing the derivatization directly in the biological material, the accuracy of the GC—ECD method if applied to analysis of, for example, other human tissues or biological material from other species should be evaluated by the GC—SIM method.

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