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Note

Gas chromatographic–mass spectrometric assay for busulfan in biological fluids using a deuterated internal standard

G. VASSAL, M. RE and A. GOUYETTE*

Clinical Pharmacology Unit (UA147 CNRS, U140 INSERM), Institut Gustave-Roussy, Rue Camille-Desmoulins, 94805 Villejuif Cedex (France)

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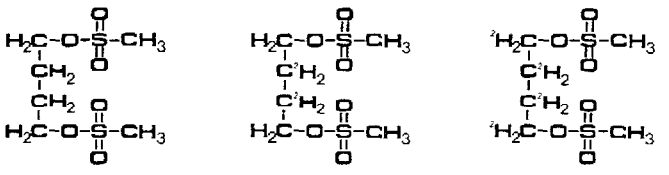
Busulfan (1,4-butanediol dimethanesulphonate, Fig. 1a), a bifunctional alkylating agent, is administered at high doses (16 mg/kg) in combined chemotherapy followed by bone marrow transplantation. High-dose busulfan gives a dose-related antitumour effect in acute leukaemias [1] and solid tumours [2] and is used as a myelosuppressive agent before allogeneic or syngeneic bone marrow engraftment in immunodeficiencies or inborn metabolism errors [3,4].

Recently, several methods of busulfan determination in plasma have been described: gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring (SIM) [5], GC with electron-capture detection (ECD) [6] and high-performance liquid chromatography (HPLC) with UV detection [7]. Of these, only the GC–MS assay could be used for pharmacokinetic studies with busulfan administered orally at conventional doses (2–6 mg per day). Metabolic studies using radioactive compounds were carried out in humans [8,9], and busulfan metabolites were identified in rat urine [10] and in bile [11] very recently.

In this paper, we describe another method to assay busulfan by GC–MS with



a b c

Fig. 1. Structures of busulfan (a), busulfan-d4 (b) and busulfan-d8 (c).

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SIM, using stable isotopes. For this purpose, two deuterated analogues were synthesized: [2,2,3,3-^{2}H]-1,4-butanediol dimethanesulphonate or busulfan-d4 (Fig. 1b) and [1,1,2,2,3,3,4,4,-^{2}H]-1,4-butanediol dimethanesulphonate or busulfan-d8 (Fig. 1c), as potential internal standards. This technique allows a specific and highly sensitive assay of busulfan in human biological fluids.

EXPERIMENTAL

Materials

Crystalline busulfan was kindly provided by the Laboratoire Techni-Pharma (Monaco). Sodium iodide, [2,2,3,3-^{2}H]-1,4-butanediol, [1,1,2,2,3,3,4,4-^{2}H]-1,4-butanediol, methanesulphonic anhydride and pyridine were obtained from Aldrich (Strasbourg, France), and dichloromethane, acetone and ethyl acetate were purchased from Merck (Darmstadt, F.R.G.).

Synthesis of busulfan-d4 and busulfan-d8

Methanesulphonic anhydride (40 mmol) dissolved in dry dichloromethane (40 ml) was carefully added to a stirred mixture of [2,2,3,3-^{2}H]-1,4-butanediol (20 mmol) in pyridine (40 ml). This exothermic reaction was carried out at 4°C, under a stream of nitrogen. The mixture was left overnight under stirring at 25°C. The solvents were evaporated and the residue was washed with distilled water to remove any trace of pyridine, then filtered to obtain a white precipitate. This solid was dissolved in the minimal amount of acetone and crystals were obtained at 4°C (yield 25%). The compound was identified by direct-probe fast-atom bombardment (FAB) mass spectral analysis and was characterized by its quasi-molecular ion, MH^+ at *m/z* 251.05 ($C_6H_{11}{}^2H_4S_2O_6 = 251.056$).

Busulfan-d8 was synthesized from [1,1,2,2,3,3,4,4-^{2}H]-1,4-butanediol by the same procedure (yield 40%) and identified by FAB mass spectral analysis: MH^+ at *m/z* 255.11 ($C_6H_7{}^2H_8S_2O_6 = 255.081$).

GC–MS system

The GC analysis was performed using a Hewlett-Packard 5790 instrument equipped with an injection splitter (split ratio, 1:100). A CP Sil5CB WCOT fused-silica capillary column (25 m×0.23 mm I.D.) with a film thickness of 0.13 μm (Chrompack, Middelburg, The Netherlands) was used. The carrier gas was helium with an inlet pressure of 1.0 bar. The instrument was operated with an injector temperature of 220°C and an oven temperature gradient of 30°C/min from 60 to 150°C, then maintained at 150°C for 6 min.

MS analyses were carried out with a VG Analytical 70-250 instrument (VG Instruments, Le Chesnay, France) equipped with an electron-impact (EI) source. The instrument resolution was set at 1000. The source was heated at 250°C and the accelerating voltage was 6000 V. The instrument was calibrated daily using perfluorokerosene. Busulfan was treated with sodium iodide, and the monitored ions were focused at *m/z* 182.9671 for transformed busulfan, *m/z* 186.9922 for transformed busulfan-d4 and *m/z* 191.0173 for transformed busulfan-d8. These

positive ions correspond to the M − 127 fragments arising from the loss of one iodine atom.

Conversion of busulfan into 1,4-diiodobutane

The conversion of busulfan and its analogues was carried out according to the method descibed by Ehrsson and Hassan [5] with the following procedure.

Busulfan assay in biological fluids

Plasma samples (1 ml) containing the drug were mixed with 100 μl of busulfan-d4 (internal standard) solution in acetone (2.5 μg/ml) and extracted with ethyl acetate (6 ml) for 10 min using a mechanical shaker (140 strokes/min). The organic phase was separated and evaporated to dryness. After addition of ethyl acetate (2 ml) and 4 *M* sodium iodide in water (1 ml), conversion was carried out under stirring at 70 °C, for 30 min. The organic phase was washed with distilled water (1 ml) and evaporated to dryness. After addition of acetone (20 μl), an aliquot (0.2–0.5 μl) was removed for GC–MS analysis. Other biological fluids [cerebrospinal fluid (CSF) and pleural effusion fluid] were treated with the same protocol.

RESULTS

Conversion of busulfan into 1,4-diiodobutane

The conversion of busulfan and its deuterated analogues into iodinated compounds proceeds in two steps: first, conversion of busulfan into 4-iodo-1-butanol methanesulphonate, then conversion into 1,4-diiodobutane. To obtain a quantitative yield, conversion had to be carried out with 4 *M* sodium iodide at 70 °C for 30 min.

GC–MS analysis of 1,4-diiodobutane, 1,4-diiodobutane-d4 and 1,4-diiodobutane-d8

Diiodobutane, diiodobutane-d4 and diiodobutane-d8 had excellent GC properties giving symmetric peaks with very close retention times: 3.55, 3.53, 3.52 min, respectively (Fig. 2). There was a small, but expected, isotope effect. Retention times are reproducible with a standard deviation of less than ±0.02 min (5.6%). This method allows detection of 10 pg injected or 0.5 ng/ml. The relative standard deviation was ±4% at 100 ng/ml ($n=9$) and ±5% at 10 ng/ml ($n=7$).

Linearity

For pharmacokinetic studies, the busulfan-d4 derivative was chosen as the internal standard, since it has the same physicochemical properties as the parent drug. Standard curves were obtained from water or plasma, using the method described. The ratio of the areas under the 1,4-diiodobutane and 1,4-diiodobutane-d4 peaks was plotted against the busulfan concentration. The calibration curves were linear in a concentration range from 10 to 1000 ng/ml. To check the reproducibility, nine standard curves were prepared from human plasma spiked with busulfan in concentrations ranging from 0 to 1000 ng/ml. All of these were

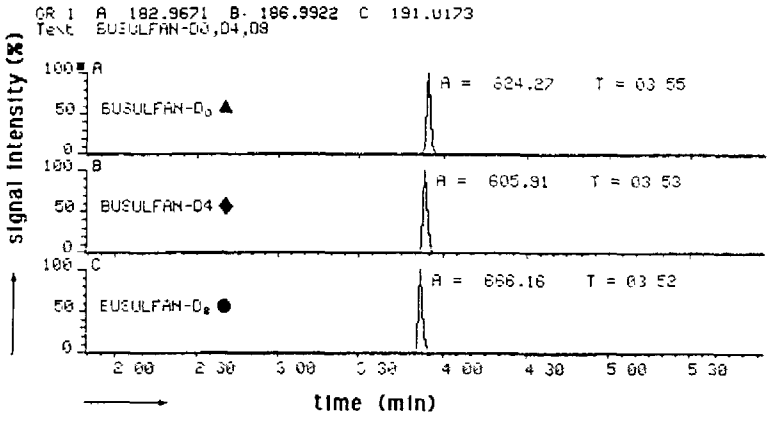

Fig. 2. GC–MS analysis of 1,4-diiodobutane (ion m/z 182.9671) (▲), 1.4-diiodobutane-d4 (ion m/z 186.9922) (◆) and 1,4-diiodobutane-d8 (ion m/z 191.0173) (●), where A = area under the curve and T = retention time.

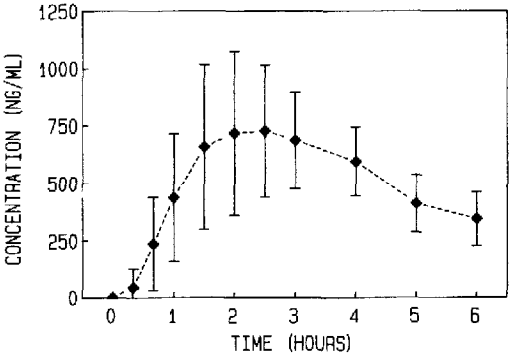

Fig. 3. Mean busulfan plasma profile in twelve children after the first oral dose of 1 mg/kg.

linear, and least-squares analysis revealed a slope of 0.0380 ± 0.0003, an intercept of 0.078 ± 0.063 and a correlation coefficient of 0.999 ± 0.001.

Plasma and CSF busulfan levels in children receiving high-dose busulfan therapy

The method was used to determine busulfan concentrations in plasma and CSF samples of twelve children treated with high-dose busulfan in combined chemotherapy for advanced malignant tumours. Children were given orally 1 mg/kg busulfan every 6 h for a total of sixteen doses over four days. Blood samples were withdrawn at different times to determine the pharmacokinetic parameters after the first and the last administration, and also during treatment to check for potential accumulation or to define the steady state. CSF samples were also obtained after the last administration. The mean maximal concentration in plasma was 803 ± 228 ng/ml and observed 92 to 255 min after dosing (see Fig. 3). The drug could also be detected in CSF where concentrations ranged between 215 and 881 ng/ml. Overall, the CSF to plasma ratio was 0.95 (range, 0.5–1.4) [12].

DISCUSSION

Unchanged busulfan has been measurable in human plasma only since 1983, when Ehrsson and Hassan described the first GC methods [5,6]. GC–ECD is the most sensitive of these assays, but cannot be used because of interfering peaks in the chromatograms [6]. GC–MS with SIM is less sensitive but gives high specificity [5]. These two methods used 1,5-pentanediol dimethanesulphonate as the internal standard. Because of its structure, 1,5-pentanediol dimethanesulphonate is more lipophilic than busulfan and its partition coefficient between aqueous and organic phase is quite different [6]. The two compounds are not extracted and converted into diiodoalkanes to the same extent. A third method using HPLC and derivatization of busulfan to 1,4-bis(diethyldithiocarbamoyl)butane seems to be easier but is less sensitive, limiting its usefulness for high-dose therapy [7].

The method described in this paper is derived from that of Ehrsson and Hassan [5]. We have used a busulfan deuterated analogue, busulfan-d4, as an internal standard because it undergoes the same processes as the parent drug during extraction (partition coefficient), conversion into diiodobutane and GC–MS analysis (only a very slight isotope effect was noticed on the retention time). Furthermore, the use of these stable isotopes is at present applied to the study of busulfan metabolism in animals and in paediatric patients. In fact, the dose-limiting effect of high-dose busulfan is the bladder toxicity, probably related to the urinary excretion of reactive metabolites, as with cyclophosphamide biotransformation products [13].

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