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Note

Measurement of busulfan in plasma by high-performance liquid chromatography

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Busulfan (1,4-butanediol dimethanesulfonate) (Fig. 1a) is the drug most frequently chosen for treatment of chronic myelogenous leukemia [1]. In recent years, busulfan has also been used as an alternative to total body irradiation in preparative regimens for bone marrow transplantation [2–7]. The usefulness of busulfan in such preparative regimens arises from both its antitumor effect and its potent hematopoietic stem cell toxicity [8–10].

Although this alkylating agent has been in clinical use since the 1950s, relatively little has been reported concerning its pharmacokinetic properties. Most previous reports of its metabolism in man have described the clearance of radioactively labelled busulfan [11]. Recently, Ehrsson and Hassan [12] have determined the kinetics of busulfan in man using a sensitive gas chromatographic–mass spectrometric (GC–MS) technique with selected-ion monitoring (SIM) to quantitate busulfan concentrations [12]. In view of the wide use of this drug, we felt that an alternative assay utilizing high-performance liquid chromatography (HPLC) technology could prove useful. In this report we describe a convenient, precolumn derivatization HPLC assay for busulfan. This assay has been used to study the solubility, stability and protein binding of busulfan in plasma and to determine plasma busulfan concentrations in patients undergoing high-dose busulfan therapy for advanced cancer.

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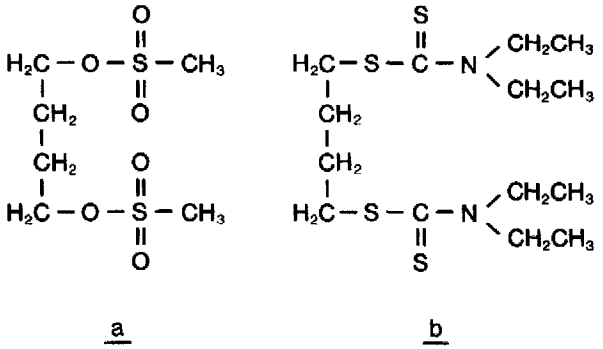

a b

Fig. 1. Structures of (a) busulfan and (b) 1,4-bis(diethyldithiocarbamoyl)butane.

EXPERIMENTAL

Materials

Crystalline busulfan was a gift of the Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). Sodium diethyldithiocarbamate was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals and solvents were HPLC grade. Centrifree micropartition units were obtained from Amicon (Danvers, MA, U.S.A.) (molecular mass cutoff 20 000). EDTA- or citrate-anticoagulated plasma was obtained from human volunteers. Samples of blood from patients receiving high-dose busulfan were obtained as part of a study performed at Duke University Medical Center. Informed consent was obtained from all patients.

1,4-Bis(diethyldithiocarbamoyl)butane

Sodium diethyldithiocarbamate (3.83 g, 17.04 mmol) was dissolved in 200 ml of distilled, deionized water. The solution was washed with one volume of chloroform to remove chloroform-soluble impurities. Busulfan (198 mg, 0.81 mmol) was added to the diethyldithiocarbamate solution. The reaction was allowed to progress for 1.5 h at 50°C and then extracted twice with one volume of chloroform. The chloroform was evaporated under a stream of glass wool-filtered air and a yellow solid was obtained. The solid was dissolved in 110 ml of methanol, filtered to remove insoluble impurities and recrystallized by the addition of water. After filtration, washing with water and drying, 47.5 mg (16% yield) of large white crystals were obtained. The product was identified as 1,4-bis(diethyldithiocarbamoyl)butane (Fig. 1b) by elemental analysis (theoretical: C, 47.68; H, 8.00; N, 7.95; S, 36.37%; actual: C, 47.95; H, 8.08; N, 7.60; S, 36.09%) and NMR (C^2HCl_3): δ 1.3 ppm (t, 12, $J=6.5$ Hz, NCH_2CH_3), 1.7–2.0 ppm (m, 4, $CH_2CH_2CH_2CH_2$), 3.2–3.5 ppm (m, 4, CH_2CH_2S) and 3.6–4.1 ppm (m, 8, NCH_2CH_3). The product has a melting point of 79–80°C and UV absorbance maxima at 278 and 252 nm with a minimum at 264 nm. The extinction coefficients at 252, 264 and 278 nm are 18 400, 14 333 and 18 700, respectively.

Derivatization of plasma busulfan

For total plasma busulfan levels, plasma samples (0.3 ml) containing the drug were mixed with two volumes (0.6 ml) of methanol and incubated at −20°C for

20 min. Following centrifugation (1500 *g*, for 10 min), 0.6 ml of supernatant was mixed with 0.15 ml of 5% diethyldithiocarbamate and 0.6 ml of 100 m*M* ammonium acetate (pH 5.5). Immediately after combining the reagents, the derivative was extracted into 1.5 ml ethyl acetate. The phases were separated using an IEC table top centrifuge at a setting of 3 for 1.5 min. A 1-ml aliquot of the ethyl acetate extract was lyophilized. To determine bioavailable busulfan levels, busulfan not bound to plasma protein, plasma (1 ml) containing busulfan was ultrafiltered using a Centrifree micropartition unit. Following ultrafiltration, 0.1 ml of plasma ultrafiltrate was mixed with 0.2 ml of methanol, 0.15 ml of 5% diethyldithiocarbamate and 0.6 ml of 100 m*M* ammonium acetate (pH 5.5). The derivative was extracted and dried as for total plasma busulfan levels.

Liquid chromatography

HPLC was performed using a Spectra-Physics solvent delivery system and a Waters μBondapak C_{18} column (10-μm particles, 30 × 0.39 cm). The mobile phase consisted of a solution of methanol–water (80:20, v/v), and the flow-rate was set at 1.0 ml/min. A Waters WISP 710B autosampler was used. Detection was achieved with an Isco variable-wavelength detector at 251 nm and a sensitivity setting of 0.01 a.u.f.s. The derivative was redissolved in 0.2 ml methanol prior to HPLC analysis.

RESULTS

Derivatization of busulfan by diethyldithiocarbamate

In aqueous solution, busulfan (Fig. 1a) reacts with diethyldithiocarbamate to form 1,4-bis(diethyldithiocarbamoyl)butane (Fig. 1b). This busulfan derivative has been identified by elemental analysis and NMR spectroscopy. The high extinction coefficient of this compound ($1.84 \cdot 10^4$ at 252 nm) makes it particularly suitable for UV detection following HPLC resolution. The derivative is stable indefinitely when dried and for more than 6 h in aqueous solution at 21 °C. It is, however, unstable at 36 or 50 °C, with half-lives of approximately 1 h and < 15 min, respectively.

Derivatization of plasma busulfan by diethyldithiocarbamate and extraction of the derivative

Derivatization by diethyldithiocarbamate takes place in methanol-deproteinized plasma or plasma ultrafiltrates for samples containing 0.125–20 μ*M* drug. The reaction was essentially instantaneous at 21 °C and pH 6.9. Under these conditions, derivatization was nearly quantitative (97.5%). Although the derivative is stable at 21 °C, with continued incubation of the derivatization reaction, materials were formed that interfered with subsequent UV detection of the busulfan derivative. Greatest sensitivity was therefore obtained if the busulfan derivative was extracted immediately following derivatization. Ethyl acetate was found to reliably and quantitatively (> 99%) extract 1,4-bis(diethyldithiocarbamoyl)-butane from plasma over the range 0.125–20 μ*M*.

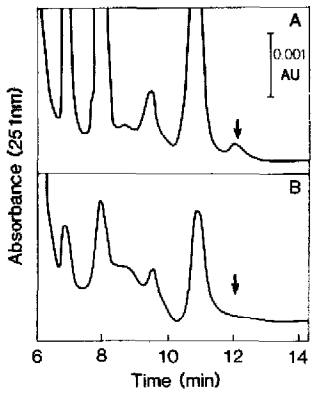

Fig. 2. HPLC profiles of diethyldithiocarbamate-derivatized plasma with (A) and without (B) busulfan. (A) 0.5 μM drug; (B) no drug. The arrow indicates the time of elution of the busulfan derivative 1,4-bis(diethyldithiocarbamoyl)butane.

Liquid chromatographic analysis

Following the derivatization, extraction and lyophilization steps, the derivative was dissolved in methanol and chromatographed by reversed-phase HPLC. 1,4-Bis(diethyldithiocarbamoyl)butane eluted at 12.0 min (Fig. 2) and was readily quantitated by peak height at 251 nm. Detection limit was routinely 0.2 μmol and occasionally as low as 0.125 μmol. Human drug-free plasma produced no interference at this retention time.

Assay linearity

A standard curve was prepared with fifteen plasma samples containing busulfan concentrations ranging from 1 to 20 μM. Derivatization and HPLC analysis were performed as described above. Over this concentration range, peak heights were linearly related to drug concentration. Least-squares analysis revealed a slope of 1.957, an intercept of 0.128 and a correlation coefficient of 0.9982. Sample variation from the regression line was an average of 5.7% with one standard deviation of the regression line of 10.3%.

Solubility and stability of busulfan in plasma

Busulfan is only sparingly soluble in water and must therefore be administered orally. It was of interest to determine the solubility of busulfan in water and plasma. Solid busulfan was slowly added to plasma or water at 22°C until the samples were saturated. After centrifugation, an aliquot of the supernatant was diluted to the appropriate concentration range and assayed as above for busulfan. Solubility of busulfan was found to be 413 μM in water and 396 μM in plasma.

The assay described above was used to determine the stability of busulfan in frozen samples of plasma. Plasma samples were spiked with 0.5–20 μM busulfan and stored at −20°C. Following 0, 16 or 57 days of storage, the samples were thawed and the busulfan concentration was determined as above. Levels of busulfan in stored samples were identical to levels in plasma analyzed immediately after the addition of busulfan (±5%).

The stability of busulfan in solution in buffer or plasma was also determined

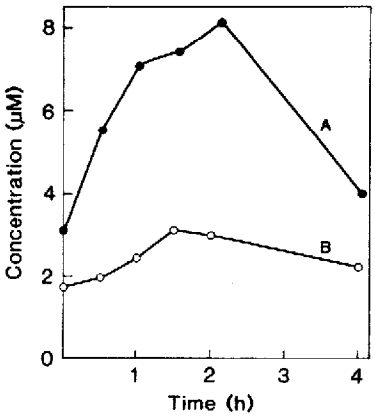

Fig. 3. Time course of high-dose busulfan in man. Blood samples were obtained following dose 9 of 1 mg/kg oral busulfan from patient A (●) or patient B (○) and plasma levels of busulfan determined.

using this method. Busulfan at 25 μM in either buffer (50 mM phosphate–0.15 M sodium chloride at pH 7.4) or human plasma was incubated at 0, 22 or 37 °C for periods up to 22 h. Samples in buffer were stable (< 15% decomposition) for more than 22 h. Samples in plasma were stable at 0 and 22 °C, but at 37 °C decomposed with a half-time of approximately 14 h.

Protein binding

To determine what fraction of plasma busulfan was reversibly bound to protein, samples of human plasma were spiked with 1–20 μM drug and an aliquot of each sample was ultrafiltered. Concentrations of the drug in ultrafiltered samples were determined as described above and compared with samples prepared by spiking ultrafiltered human plasma with 1–20 μM busulfan. The concentration in ultrafiltered samples was 45.1 ± 8.5% of that in non-filtered samples indicating that a significant amount of the drug in plasma was bound to macromolecules of molecular mass > 20 000. The binding of busulfan to plasma proteins is abolished by the addition of as little as 2% methanol (v/v) to the plasma prior to ultrafiltration.

Plasma busulfan in patients receiving high-dose busulfan

The above assay has been used to determine the concentrations of busulfan in plasma of patients receiving high-dose busulfan as treatment for advanced cancer. Patients received 1 mg/kg busulfan by mouth every 6 h for a total of sixteen doses. At the indicated times (Fig. 3) blood samples were obtained and levels of total plasma busulfan determined. Busulfan was easily detected and quantitated in these plasma samples.

DISCUSSION

The assay described here provides a convenient method for the quantitation of busulfan in plasma using reversed-phase HPLC and UV detection. The only pre-

viously described method for quantitating busulfan in clinical samples [12] requires GC–MS with SIM, a technology not readily available to many investigators. We have demonstrated that the HPLC assay is sufficiently sensitive to study busulfan kinetics during high-dose therapy (1 mg/kg per dose) such as is administered in preparation for bone marrow transplantation. Additional details of the pharmacokinetics of high-dose single-agent busulfan utilizing this assay will be reported along with a description of the clinical course of patients treated in this fashion [13]. This HPLC assay should also be useful in biochemical and animal studies of busulfan. However, the HPLC assay is not sufficiently sensitive for studies of busulfan kinetics in man when administered at conventional doses of 2–6 mg per day. Studies of conventional-dose busulfan must still rely on the use of GC–MS with SIM.

The assay has also been used to study the properties of busulfan in vitro, and these properties provide some insight into the biologic properties of this drug. Busulfan is only sparingly soluble in water and must be administered orally. However, the peak plasma levels of busulfan with high-dose therapy ($\leq 10\ \mu M$) are much less than that of plasma saturated with busulfan (approximately 390 μM). It has been reported that busulfan crystals can be found in the cells of livers of patients following high-dose busulfan therapy [14]. If such crystals are indeed busulfan, then busulfan must be greatly concentrated by uptake into these cells or, during absorption of busulfan from the gut, concentrations of busulfan must be transiently much greater in the hepatic circulation than those achieved in the peripheral circulation. The in vitro studies have also determined that approximately 55% of busulfan in plasma samples exists in a form reversibly bound to proteins. Such binding will reduce the amount of bioavailable drug. A previous study of busulfan binding to plasma proteins, as measured by equilibrium dialysis, did not detect reversible binding to proteins [15]. However, such binding is easily disrupted by the presence of even small amounts of organic solvents such as methanol, commonly used as solvents for busulfan. The presence of small amounts of such solvents during equilibrium dialysis may account for the lack of observed binding.

Busulfan is relatively stable in plasma at physiologic temperatures. The 14 h half-life under these conditions is much longer than the 20–25 min half-life of an alkylating agent such as carmustine incubated under the same conditions. The long in vitro half-life [16] suggests that active metabolism rather than simple decomposition accounts for most of the clearance of the drug in vivo. The availability of an HPLC assay for this drug should assist in defining the exact metabolic processes involved in handling of this drug.

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