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Note

Quantitation of busulfan in plasma by high-performance liquid chromatography using postcolumn photolysis

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Busulfan [Myleran™, 1,4-bis(methanesulphonyloxy)butane], a straight-chain bifunctional alkylating agent (Fig. 1), has been used since the 1950s in low doses (0.1 mg/kg per day) especially for the treatment of chronic myelogenous leukemia [1].

Since the late 1970s, busulfan in high doses (oral application of 1 mg/kg) every 6 h for sixteen doses), followed by application of cyclophosphamide, has been widely used in the preparative regimen for bone marrow transplantation. Administration of extremely high doses of cytotoxic drugs as preparation for syngeneic, allogeneic and autologous bone marrow transplantation has led to highly encouraging clinical results. The continued expansion of bone marrow transplantation programmes, however, will depend on the development of means of overcoming such non-haematological toxicities as hepatic veno-occlusive disease (VOD) of the liver.

VOD occurs in *ca.* 10–20% of patients undergoing bone marrow transplantation [2–9]. VOD causes 10% of transplantation-related deaths, making it the third leading cause of death in allogeneic graft recipients and the second leading cause in patients receiving autologous grafts. New investigations revealed that

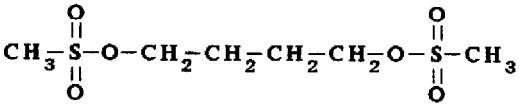

Fig. 1. Structure of busulfan

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VOD is probably related to the total amount of busulfan in the plasma after the first dose during this preparative regimen [10].

In this context analytical techniques are urgently required to enable the monitoring of the plasma level of busulfan after the first application as fast as possible. This would allow the adjustment of the dose at an early point in time in this high-dose therapy. The adjusted dose could lower the risk of undesirable toxic side-effects.

Several methods for busulfan determination in plasma have been described: gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring (SIM) [12,13]; GC with electron-capture detection (ECD) [11,14]; and high-performance liquid chromatography (HPLC) with UV detection [10,15]. These methods are indeed very sensitive but require either costly equipment (GC–MS) or involve time-consuming and elaborate sample preparation procedures [10,14,15] unsuitable for routine analysis.

This paper describes a new analytical method for the determination of busulfan in plasma: it is an adaptation of an established derivatization reaction originally developed for GC analysis [11–13]. The assay, developed for application in clinical routine, utilizes an isocratic HPLC system modified by the addition of an inexpensive photolysis unit for postcolumn derivatization.

The nucleophilic substitution of the methanesulphonate moieties of busulfan by iodide in a precolumn derivatization leads to 1,4-diiodobutane, which is chromatographed and detected after on-line postcolumn photolysis. The photochemical reaction leads to the photodissociation of the carbon–iodine bonds with formation of iodide ions [16,17]. The subsequent UV detection of the iodide ions is performed at 226 nm.

The new HPLC assay has a very high selectivity for busulfan, which is comparable with the selectivity achievable after GC separation using SIM-MS or ECD. The detection sensitivity is sufficent to allow evaluation of pharmacokinetic data in the multi-dose preparative regimen for bone marrow transplantation.

EXPERIMENTAL

Reagents and chemicals

Busulfan (98% purum), 1,4-diiodobutane (99% purum), *n*-heptane, methanol and 2-methoxyethanol (HPLC grade) were purchased from Aldrich (Steinheim, F.R.G.) and acetone (Uvasol) and sodium iodide (99.5%) from Merck (Darmstadt, F.R.G.), and they were used without further purification All water used was deionized and filtered with a Milli-Q™ water purification system (Milford, MA, U.S.A.).

HPLC apparatus

The chromatographic system consisted of an analytical HPLC pump Model 2200 (Bischoff, Leonberg, F.R.G.) and a universal liquid chromatograph injector

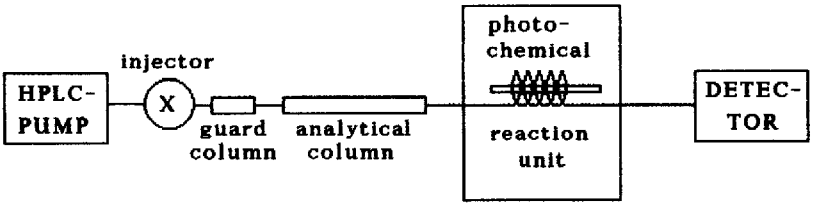

Fig. 2. HPLC apparatus with photochemical postcolumn derivatization unit.

Model U6K (Millipore/Waters, Eschborn, F.R.G.). For injection a 25-μl syringe (Hamilton, Reno, NV, U.S.A) was used. Detection was carried out with a Spectrachrom 200 variable-wavelength spectrophotometer (Spectra-Physics, Darmstadt, F.R.G.). The signal was calculated by means of a 2221 integrator (Pharmacia-LKB, Freiburg, F.R.G.).

The chromatographic column system consisted of a 5-μm LiChrosorb CN analytical column (250 mm × 4 6 mm I.D.) and a guard column of 5-μm LiChrosorb CN (50 mm × 4.6 mm I.D.), purchased from Grom (Herrenberg, F.R.G.).

For postcolumn derivatization a photochemical reaction unit was placed between the column and the detector (Fig. 2). It consisted of an aluminium box (30 cm × 34 cm × 15 cm) equipped with an electric fan. On the inner side of the front a germicidal lamp type G8T5 (GTE Sylvania, Danvers, MA, U.S.A.), inserted in a knitted PTFE reactor, was placed. The photochemical reactor was handknitted cylindrically with eight right stitches using five needles of 3 mm diameter. The resulting reactor had a height of 9 cm and an I.D. of *ca.* 1.8 cm The I.D. of the reactor tube was 0.8 mm, and the total volume was 2.4 ml. To achieve derivatization the eluate was irradiated as it flowed through the reactor.

Chromatographic conditions

The separation of the busulfan derivative 1,4-diiodobutane was achieved with an isocratic solvent system of water–methanol (80:20) at a flow-rate of 1 ml/min. The mobile phase was filtered by a Millipore filter (GV 0 22 μm) and degassed by helium flow. The optimal detection wavelength was 226 nm at a sensitivity of 0.002 a.u.f.s. Under these conditions, the retention time was 16.5 min.

The extremely selective and sensitive determination of busulfan is demonstrated by comparison of lamp-on and lamp-off chromatograms of a patient's plasma sample (Fig. 3A and B).

Standards

Busulfan plasma standards were prepared by the following procedure: 200 μl of a busulfan stock solution (50 μg/ml in acetone) were placed in a clean glass tube and evaporated to dryness under a stream of nitrogen; 2 ml of drug-free pooled plasma were added and busulfan was redissolved by sonication for 2 min. This

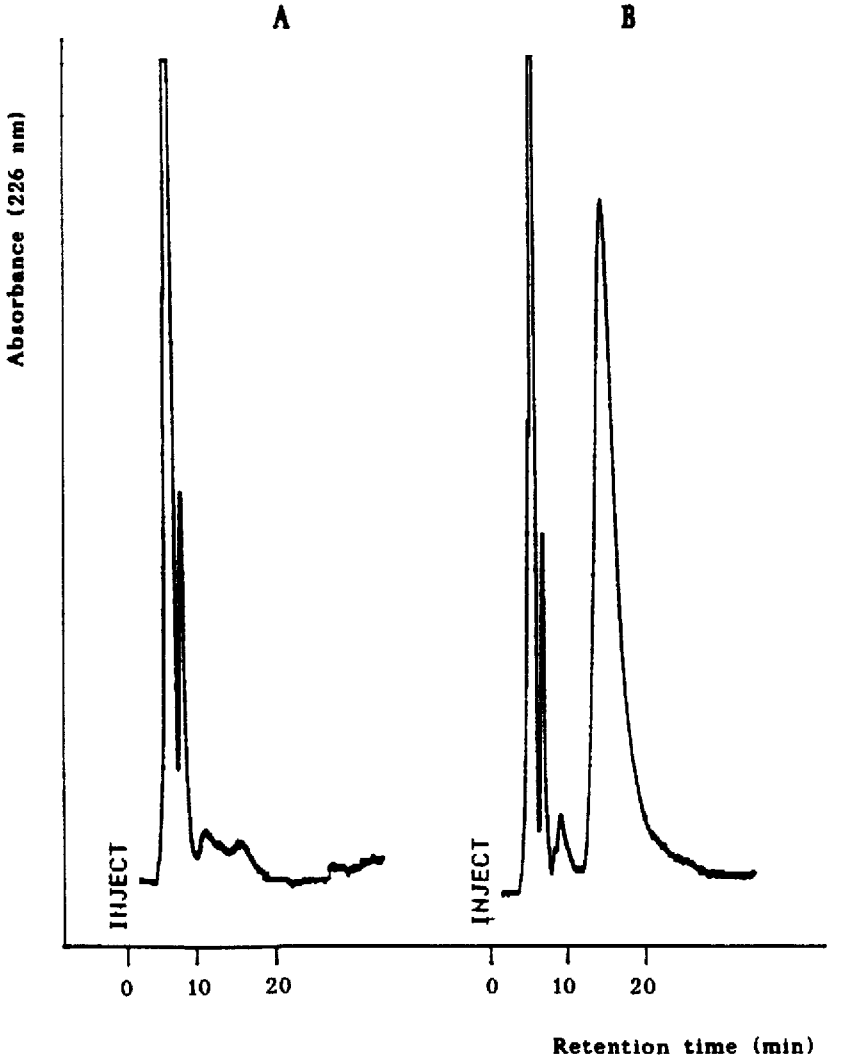

Fig 3 Demonstration of the high selectivity of the photolytic HPLC analysis of a plasma sample (containing 880 ng/ml) of a patient treated with busulfan prior to bone marrow transplantation (taken 90 min after administration of the drug) (A) Without UV irradiation, (B) with UV irradiation

stock solution was further diluted with plasma to achieve the final desired plasma standard concentrations from 50 ng/ml to 5 μg/ml.

For determination of the recovery, standards of 1,4-diiodobutane were prepared in 2-methoxyethanol over the same molar concentration range.

Sample treatment

Heparinized blood samples were collected from patients for 6 h after oral

application of the drug. The samples were immediately chilled in ice-water. After centrifugation at 4°C, the plasma was removed and immediately assayed or stored at −20°C until use. At −20°C busulfan plasma samples were stable for up to six months [10].

Extraction procedure

A Vac Elut™ system equipped with Bond Elut™ 1-ml C_8 cartridges (Analytichem International, Handelsgesellschaft) was used for sample clean-up. An aliquot of 1 ml of plasma was passed through a cartridge, which was sequentially preconditioned by washing with 1 ml of methanol and 3 ml of water. After the plasma had passed through the cartridge, it was washed with 2 ml of water and dried. Then 1 ml of methanol was used to elute the drug by centrifugation into a glass reaction vial.

Derivatization procedure

Immediately after extraction, 1 ml of 4 *M* aqueous sodium iodide solution and 400 μl of *n*-heptane were added to the methanolic extract in the reaction vial. The vials were tightly closed and the mixture was heated with stirring at 70°C for 40 min in a water-bath. After cooling to room temperature, 350 μl of the upper layer were removed and centrifuged in an Eppendorf microcentrifuge at 12 000 *g* to ensure total separation of aqueous and organic phases. Then 250 μl of the upper (organic) layer were transferred to a microcentrifuge tube, containing 100 μl of 2-methoxyethanol. *n*-Heptane was evaporated without heating in a Speed Vac concentrator for 8 min. An aliquot of 20 μl of the remaining extract was injected into the liquid chromatograph.

RESULTS

Quantitative analysis

Quantitation was performed according to the external standard method by plotting the peak area against known concentrations of spiked plasma standards. Linearity was demonstrated from 50 ng/ml to 5 μg/ml of plasma at eleven known concentrations. The correlation coefficient was 0.999. The detection limit for busulfan in plasma was 20 ng/ml, based on a signal-to-noise ratio of 3. Drug-free pooled plasma samples showed no peaks that would interfere with 1,4-diiodobutane.

Recovery

The recovery was calculated by comparing the peak areas of the busulfan derivative in plasma after extraction and derivatization to equivalent amounts of 1,4-diiodobutane injected directly into the HPLC system. The extraction and derivatization efficiency from plasma was 83%.

TABLE I

PRECISION OF HPLC–UV PHOTOLYTIC ASSAY FOR BUSULFAN IN PLASMA

Assay	Concentration (ng/ml)	*n*	C V (%)
Intra-day	250	10	2 7
Inter-day	125	7	11.5
	1250	7	7.6

Reproducibility

The reproducibility was evaluated after repeated injections of the same extract (intra-day assay) and after extraction and derivatization of two given concentrations of busulfan (inter-day assay). The results are given in Table I.

Pharmacokinetic results

To prove its applicability the method was tested on one patient's blood samples, after he received the first of sixteen doses of 100 mg of busulfan. The result is shown in Fig. 4. The pharmacokinetic curve fitted a two-compartment model with the maximal plasma concentration at 33.6 min and an elimination half-live of 3.2 h.

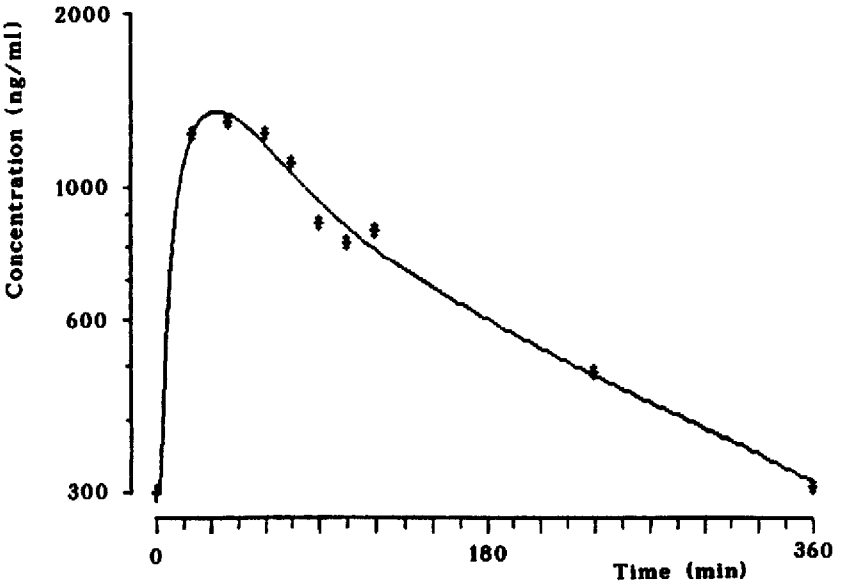

Fig. 4. Concentration–time curve of busulfan in a patient's plasma sample after the first oral dose of 1 mg/kg Subsequent doses were administered at intervals of 6 h.

DISCUSSION

In view of the high mortality associated with VOD we decided to investigate possible reasons for its appearance. As suggested in the literature, treatment with cytotoxic agents seems to be highly correlated with the development of hepatic necrosis, including VOD. Information about the individual drug disposition and the pharmacokinctic behaviour during an carly phase of the therapy could be helpful in overcoming the toxic side-effects of the regimen used.

The literature search revealed that most of the analytical methodologies published do not allow the determination of drug levels in plasma in a routine laboratory, although all methods exhibited sufficient sensitivity.

Two assays, utilizing GC–ECD and HPLC, used derivatization of busulfan with tetrafluorothiophenol and thiophenol, respectively, but required more than 2.5 h [10,14] for sample preparation only. An alternative HPLC method involved lyophilization of the extracts and produced complex samples with partially interfering substances [15]. Furthermore, the low concentration limit (250 ng/ml) of the linear range of the calibration curve could restrict the clinical application of this method.

Only methods using derivatization with sodium iodide and subsequent GC separation showed short and simple sample preparation [11–13], allowing their routine application. Unfortunately, the high sensitivity of these methods is coupled with expensive equipment, such as GC–MS or ECD systems, not in routine use in most centres. The development of an alternative routine HPLC method would allow the application of less costly and probably more available equipment.

The assay described here provides a convenient method for the quantitation of busulfan in plasma. It features easy and fast sample handling, a good sensitivity and excellent selectivity. Among the previously employed derivatization reactions, the nucleophilic substitution of the methanesulphonate moiety with iodide anions is the most selective one because the extraction of the 1,4-diiodobutane produced can be effected with *n*-heptane, which yields extracts without interfering compounds. Subsequently, isocratic chromatographic conditions were sufficient to obtain good separations. Interestingly we observed more than double yields of recovery of the busulfan derivative if the plasma samples were extracted by solid phase prior to derivatization. This result was unexpected because it contrasts with the results of Hassan and Ehrsson [11], who found 91 ± 3.2% recovery of the reaction product performing the reaction without prior extraction using the same reaction conditions Transfer of 1,4-diiodobutane from *n*-heptane into a solvent suitable for reversed-phase HPLC analysis occurs by vacuum centrifugation without control of pressure.

Because 1,4-diiodobutane possesses no chromophoric system suitable for sensitive HPLC detection, a second derivatization was necessary. On-line postcolumn photochemical reaction derivatization is the most convenient technique for this

purpose, allowing derivatization without further time-consuming sample handling [16–18]. In this way, 1,4-diiodobutane is converted into iodide anions with an efficiency of *ca.* 50%. Detection of iodide anions is possible either with UV at 226 nm or with electrochemical oxidation [16,17]. The sensitivity limit for 1,4-diiodobutane using UV detection lies at 1 ng.

The photochemical reactor can be knitted easily using PTFE tubes available for chromatographic purposes, or can be obtained commercially (Handelsgesellschaft, Frankfurt, F.R.G.). A complete photochemical reaction unit is also available (Beam Booster™). Using the commercial reactor, with 25 m tube length and 0.3 mm I.D., the same results could be achieved as found by our laboratory-made reactor.

The search for an internal standard using homologues of busulfan was unsuccessful because recoveries for these substances after solid-phase extraction were very low.

The main advantage of the HPLC technique described here is speed combined with high selectivity. Thus, the total operator time of *ca.* 1.5 h for the work-up of ten plasma samples and subsequent quantitation within 2.5 h is sufficiently short to generate analytical data on plasma level concentrations of busulfan in time to adjust the third dose to a particular disposition of the drug.

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