Journal of Chromatography, 532 (1990) 424–428
Biomedical Applications
Elsevier Science Publishers B V., Amsterdam

CHROMBIO 5442

Note

Quantitation of busulfan in plasma by high-performance liquid chromatography

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(First received January 29th, 1990, revised manuscript received July 4th, 1990)

A precolumn derivatization high-performance liquid chromatographic (HPLC) method for the measurement of busulfan (1,4-butanediol dimethanesulfonate) in human plasma was reported by Henner *et al.* in 1987 [1]. This method offered a simple and convenient alternative to the gas chromatographic and mass spectrometric techniques used previously [2–4], and was sufficiently sensitive for pharmacokinetic studies of high-dose busulfan therapy (4 mg/kg per day for four days) in bone-marrow transplant patients [1].

We offer several modifications to the method of Henner *et al.* [1]. These include simplification of the extraction/derivatization procedure, incorporation of an internal standard, and addition of a solid-phase clean-up step. The sensitivity limit is also improved by modifications to the liquid chromatographic system. With this method, plasma busulfan concentrations as low as 0.05 μg/ml (0.20 μM) can be precisely quantitated, with a lower limit of detection of 0.4 ng/ml (0.0016 μM).

EXPERIMENTAL

Materials

Busulfan was donated by Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). The internal standard, N-(2,6-difluorobenzoyl)-N′-[3,5-dichloro-4-(3-chloro-5-trifluoromethylpyridin-2-yloxy)phenyl]urea (CGA-112913), was donated by Ciba Geigy (Basel, Switzerland). Sodium diethyldithiocarbamate was obtained from Sigma (St. Louis, MO, U.S.A.) and was stored at −20°C in a dessicator. Acetonitrile, tetrahydrofuran, and methanol were HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other chemicals were reagent grade. A MicroPak-SP-C_{18} HPLC column (3 μm particle diameter, 15 cm × 0.40 cm) was purchased from Varian Assoc. (Sunnyvale, CA, U.S.A.). Solid-phase extraction columns packed with C_{18} stationary phase (1-ml

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volume, Bakerbond spe®) were purchased from J.T. Baker, (Phillipsburg, NJ, U.S.A.). Polypropylene test tubes (8 ml) and 100 mm × 12 mm glass culture tubes were purchased from KEW Scientific (Columbus, OH, U.S.A.).

An isocratic HPLC pump (Varian Model 2010) fitted with a Rheodyne sample injection valve (Model 7125) was used with a variable-wavelength ultraviolet detector (Varian Model 2050) set at 278 nm. Detector output was recorded on a strip chart recorder (Model 1200, Linear Instruments, Reno, NV, U.S.A.).

Reagents and plasma standards

A 5% solution of diethyldithiocarbamate (DEDC) was prepared by dissolving 8.2 g of sodium DEDC in 100 ml of water, and was stored at 8°C when not in use. A stock solution of busulfan was prepared in ethanol at a concentration of 100 μg/ml. Serial dilution was performed to produce ethanolic spiking solutions ranging in concentrations from 2.5 to 40 μg/ml. A 30-μl volume of each solution added to 0.3 ml of plasma produced final plasma concentrations ranging from 0.25 to 4 μg/ml. The spiking solutions were stored in polypropylene test tubes at −20°C when not in use. A stock solution of internal standard (200 μg/ml in ethanol) was diluted to 25 μg/ml with methanol. This working internal standard solution was stored in a polypropylene test tube at −20°C. Stability of busulfan stock solutions were tested by spiking plasma in duplicate with solutions that had been prepared at various times over an eighteen-month period and stored at −20°C. Spiked plasma samples were processed according to the procedure described below, and measured peak-height ratios of busulfan derivative to internal standard were compared to the ratio from plasma spiked with a freshly prepared stock solution.

Extraction and chromatography

The derivatization procedure of Henner *et al.* [1] was simplified by omission of the initial protein precipitation step. Plasma (0.3 ml) was placed in an 8-ml polypropylene test tube to which 30 μl of internal standard working solution had been added. Plasma for calibration standards was also spiked with 30 μl of the appropriate busulfan spiking solution. Busulfan was derivatized by the addition of 0.15 ml of 5% DEDC followed by vortex-mixing for 10 s. Methanol (0.2 ml) was then added followed by 10 s vortex-mixing. Ethyl acetate (2 ml) was added, the mixture was briefly vortex-mixed, and the tubes were gently rocked for 10 min on a blood mixer. After centrifuging at 1500 *g* for 10 min, a 1-ml aliquot of the organic layer was transferrred to a 100 mm × 12 mm glass culture tube and evaporated to dryness (approximately 10 min) under filtered air using a heating block set at 70°C. The residue was dissolved in 0.5 ml of methanol, followed by the addition of 0.5 ml of water.

The solid-phase extraction columns were conditioned with three 1-ml volumes of methanol, followed by two 1-ml volumes of water. Particular care was taken not to allow the columns to dry out after the final water rinse. The reconstituted

extracts were slowly pulled through the conditioned columns, and the columns were rinsed with two 1-ml volumes of 50% methanol in water. The analytes were eluted from the columns into 100 mm × 12 mm glass culture tubes using two sequential 250-μl volumes of methanol.

The mobile phase was a solution of acetonitrile–water–tetrahydrofuran (55:25:20, v/v) at a flow-rate of 0.8 ml/min. Aliquots of 20 μl of each extract were injected at detector ranges of 0.005–0.08 a.u.f.s. The recorder was set at 10 mV, with a speed of 30 cm/h. Calibration curves were constructed relating peak-height ratio (busulfan derivative/internal standard) to concentrations of busulfan in calibration standards.

Analytical variables

Within-day precision and accuracy were evaluated by processing three sets of ten plasma samples containing busulfan concentrations of 0.050, 0.250, or 3.000 μg/ml, and determining the mean concentration and coefficient of variation (C.V.) for each according to the described procedure. For the 0.05 μg/ml concentration, the injection volume was doubled. The lower limit of detection (LLD) was determined as described by Anderson [5] using busulfan plasma concentrations ranging between 0.006 and 0.05 μg/ml. For these lower concentration samples, as much as 40 μl were injected at a detector range of 0.0025 with the chart recorder set at 5 mV.

Extraction recovery of the internal standard from plasma was determined by comparison of peak heights in extracts, corrected for losses in transfers, with peak heights obtained by direct injection of an unextracted solution. Elution recovery of analytes from the solid-phase extraction columns was determined by comparison of peak heights in samples taken through the columns once to those taken through twice.

RESULTS AND DISCUSSION

Resolution of the busulfan derivative and internal standard from plasma components is illustrated in Fig. 1A and B. Without the solid-phase extraction step, a large solvent front obscures the peaks corresponding to these two analytes. Retention times for the busulfan derivative and internal standard, respectively, are 4.3 and 5.6 min Although resolution was also obtained when a 5 μm particle diameter column (MicroPak-SP-C_{18}, Varian Assoc.) was tried, peak heights were approximately one half of those observed using the smaller particle diameter stationary phase. In contrast to the detection wavelength of 278 nm, peak heights of the busulfan derivative were slightly smaller (by 13%) when a 254-nm wavelength was used, while peak heights of the internal standard were 25% higher. As with the 278-nm wavelength, no interferences from plasma were seen when a 254-nm wavelength was used. We therefore conclude that a fixed-wavelength detector (254 nm) would be equally suitable for the implementation of this method.

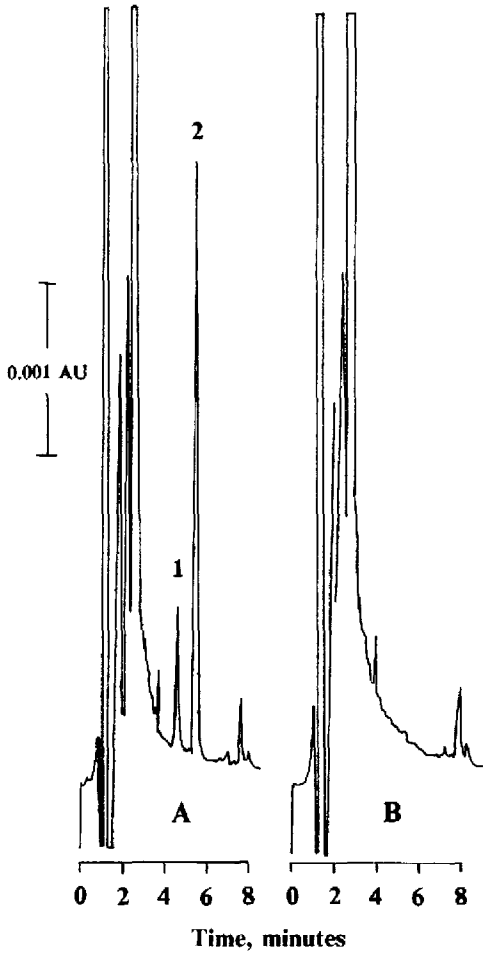

Fig 1 Chromatograms of (A) extracted plasma spiked with 0.25 μg/ml (1 0 μ*M*) busulfan and (B) drug-free plasma Peaks: 1 = 1,4-bis(diethyldithiocarbamoyl)butane; 2 = internal standard (CGA-112913).

As compared to a freshly prepared ethanolic stock solution, busulfan stock solutions stored at −20°C for eight, twelve and eighteen months were 95, 93, and 89% of the concentration in a freshly prepared solution.

The internal standard was 97% recovered from plasma using our extraction procedure. The addition of methanol to the plasma sample was found to be critical for recovery, presumably because it disrupts plasma protein binding. Without the methanol, recovery of the internal standard was 51%. The internal standard is unaffected by the addition of the derivatizing agent (DEDC), as evidenced by identical retention times of extracted and unextracted internal standard. Both the busulfan derivative and internal standard were 100% recovered from the solid-phase extraction columns. Prolonged drying of the extracts (as long as 1 h at 70°C) did not affect peak heights or peak-height ratios of the analytes.

The mean concentrations and corresponding C.V. values (in parentheses) for replicate plasma busulfan concentrations of 0.050, 0.250 and 3.000 μg/ml were 0.052 (2.8%), 0.253 (1.5%), and 3.002 (1.2%), respectively. The lower limit of detection for the busulfan derivative was 0.4 ng/ml. This low limit of detection can be mostly attributed to the sharper peaks seen with use of the small-particle-diameter column. Peak sharpness was also improved by the use of acetonitrile in the mobile phase, as compared to methanol which was used in the original method [1]. Calibration curves were linear up to busulfan plasma concentrations of 20 μg/ml and deviated significantly from linearity starting at a concentration of 40 μg/ml.

In summary, this method provides several advantages over the original method reported by Henner *et al.* [1]. The internal standard corrects for variability in transfer and injection volume measurements, thus providing excellent precision at very low concentrations. The solid-phase extraction step provides interference-free chromatograms under the chromatographic conditions used. And, finally, the modified chromatographic conditions provide improved sensitivity. This method could therefore be used for plasma busulfan measurements following first doses as well as during chronic dosing.

AKNOWLEDGEMENT

The technical assistance of Mr. Steven Baskerville is gratefully acknowledged.

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