ELSEVIER

Journal of Chromatography B, 660 (1994) 200–204

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

High-performance liquid chromatographic determination of busulfan in human serum with on-line derivatization, column switching and ultraviolet absorbance detection

Ken-ichi Funakoshi*, Kenji Yamashita, Wen-feng Chao, Masashi Yamaguchi, Takatsuka Yashiki

Takeda Analytical Research Laboratories, Ltd., Juso-Honmachi, Yodogawa-ku, Osaka 532, Japan

First received 18 February 1994; revised manuscript received 30 May 1994

Abstract

A high-performance liquid chromatographic (HPLC) method is described for the determination of busulfan in human serum using on-line derivatization and column switching. Busulfan was extracted from serum with a mixture of diethyl ether and dichloromethane. After the evaporation of the organic layer, the reconstituted residue was injected into the HPLC system and busulfan was derivatized with sodium diethyldithiocarbamate on the first short column. The back-flushed derivative was then separated on the second column. Finally, after column switching, the heart-cut fraction containing the derivative was further analysed on the third column and monitored with ultraviolet absorbance detection at 278 nm. The lower limit of quantitation in serum was 10 ng/ml.

1. Introduction

Busulfan is frequently used in high doses in the preparative regimen for bone marrow transplantation [1]. Several methods have been developed for the determination of busulfan in biological fluids, including gas chromatography–mass spectrometry (GC–MS) [2,3], GC with electron-capture detection (ECD) [1,4,5] and high-performance liquid chromatography (HPLC) [6–10]. GC–MS offers very high sensitivity but is not suitable for routine analysis. GC–ECD and HPLC are not as sensitive as GC–MS, but sensitive enough for drug monitoring in high-dose treatment. With respect to suitability for routine analysis, HPLC was considered to be the method of choice. Busulfan shows very poor absorption in the ultraviolet (UV) wavelength region. Therefore, MS [6], postcolumn photolysis [7] or precolumn derivatization with sodium diethyldithiocarbamate (DDC) [8,9] were used for the HPLC analysis. However, Kazemifard and Morgan [10] reported that this precolumn derivatization reaction was incomplete and not quantitative, and therefore was not useful for measuring busulfan concentration. We first tried to develop an HPLC method using this precolumn derivatization reaction. However, the reaction was not reproducible, as reported previously [10]. This suggested that the conditions for this reaction should be very strictly controlled, which made it difficult

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00273-8

for the reaction to be applicable in routine analysis. In this respect, on-line derivatization was considered to be hopeful, in which the reaction condition could be automatically and therefore more reproducibly controlled than in the above-described manual precolumn derivatization method [8–10].

This paper describes an HPLC method for the determination of busulfan in human serum with on-line derivatization, column switching and UV absorbance detection.

2. Experimental

2.1. Reagents and materials

Busulfan and DDC were obtained from Wako (Osaka, Japan). Diethyl ether, dichloromethane and acetonitrile were of HPLC grade (Wako). All other reagents were of analytical-reagent grade and were used as received.

2.2. Extraction from serum

To 0.5 ml of human serum were added 100 μl of water and the mixture was extracted with 5 ml of diethyl ether–dichloromethane (7:3, v/v) by shaking for 5 min, centrifuging at 1500 *g* for 5 min and freezing the aqueous layer in a dry-ice–acetone bath. The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 250 μl of water and an aliquot of 100 μl was injected into the HPLC system.

2.3. Instrument and conditions

The HPLC system consisted of three LC-6A pumps, two UV detectors (SPD-6A and SPD-10A), two FCV-2AH six-port switching valves and a SIL-6B autoinjector, all of which were controlled by an SCL-6B controller (all from Shimadzu, Kyoto, Japan). A U-228 dual-pen recorder (Nippon Denshi Kagaku, Kyoto, Japan) was used for the measurement of the peak height of the busulfan derivative. Three ODS columns were used: Inertsil ODS-80A (5-μm particle size, 10 × 4.0 mm I.D.; GL Science, Tokyo, Japan) as column 1 (C1), Inertsil ODS-2 (5-μm particle size, 150 × 4.6 mm I.D.; GL Science) as column 2 (C2) and Capcell Pak C_{18} (5-μm particle size, 150 × 6.0 mm I.D.; Shiseido, Tokyo, Japan) as column 3 (C3). Another column similar to C1 was also inserted between pump A [P(A)] and the autoinjector as a clean-up column for MP(A) (C0). The mobile phases for C1, C2 and C3 [MP(A), MP(B) and MP(C)] were as follows: 1% DDC aqueous solution as MP(A), and acetonitrile–20 m*M* potassium dihydrogenphosphate (pH *ca.* 4.6) [60:40, v/v, as MP(B) and 65:35, v/v, as MP(C)]. The flow-rates for C1, C2 and C3 were 0.5, 1.0 and 1.0 ml/min, respectively. The temperature was 25°C for all columns. UV detection was carried out at 278 nm.

2.4. Analytical system and procedure

A schematic diagram of the HPLC system is shown in Fig. 1. Busulfan in the injected sample was derivatized with DDC on C1 for 5 min with MP(A), followed by back-flushing of the deriva-

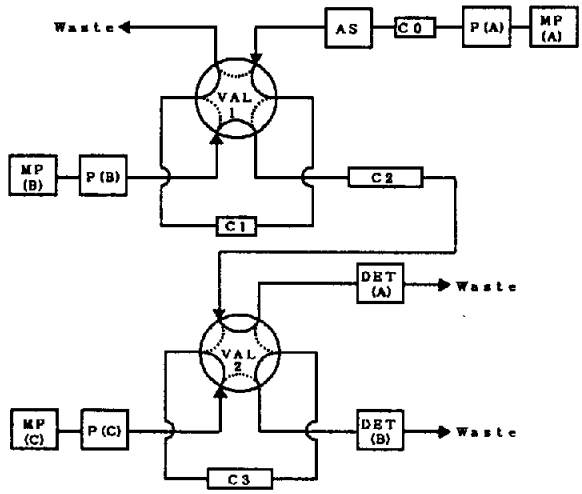

Fig. 1. Schematic diagram of the HPLC system. P(A), P(B) and P(C) = pumps A, B and C; AS = autosampler; VAL-1 and VAL-2 = six-port valves 1 and 2; C0 = clean-up column; C1, C2 and C3 = columns 1, 2 and 3; DET(A) and DET(B) = UV detectors A and B; MP(A), MP(B) and MP(C) = mobile phases A, B and C. The solid and dotted lines in the six-port valves indicate valve positions 0 and 1, respectively.

tive from C1 to C2 by switching the valve 1 position from 0 to 1. At 8 min after injection, the valve 1 position was switched back to 0 and the derivative was successively separated on C2 with MP(B). At 10 min, the eluate fraction containing the derivative was transferred from C2 to C3 by changing the valve 2 position from 0 to 1. Immediately after the elution of the derivative from C2 to C3 (at 10.6 min), the valve 2 position was switched back to 0, and the heart-cut fraction was further analysed on C3 with MP(C) and monitored by measuring the UV absorbance at 278 nm. During the procedure, P(A) was stopped from 5.1 to 32 min. The analysis for each sample was completed within 33 min. All these operations were carried out automatically by the SCL-6B controller according to the predetermined time programme.

3. Results and discussion

Busulfan shows very poor absorption in the UV wavelength region. Therefore, some kind of derivatization was thought to be required for the determination of busulfan in biological fluids. Precolumn derivatization reaction with DDC has been reported for the HPLC determination of busulfan in plasma [8,9]. However this reaction was claimed to be incomplete and not quantitative by Kazemifard and Morgan [10]. This poor reproducibility was presumed to be overcome if the conditions of the reaction were very strictly controlled. In this respect, on-line derivatization was considered to be advantageous over precolumn derivatization, because the reaction could be automatically and therefore reproducibly controlled.

Two ODS mini-columns were examined as a derivatization column (C1): Guard Pak μBondapak C_{18} (Waters, Milford, MA, USA) and Inertsil ODS-80A (5-μm particle size, 10 × 4.0 mm I.D.). The latter column was selected owing to its higher formation of the derivative, probably because of the better retainability (data not shown).

To optimize the conditions for the reaction, the concentration of the reaction solution [MP(A)] and the reaction time on C1 with MP(A) were studied. The effect of the concentration of DDC aqueous solution [MP(A)] on the formation of the busulfan derivative is shown in Fig. 2. A higher concentration led to a higher formation of the derivative. Considering the aqueous solubility of DDC and the increase in interferences in the chromatogram with higher concentration, 1% DDC aqueous solution was selected as MP(A). The investigation of the effect of the reaction time on C1 on the yield of the derivative showed that 5 min was optimum (Fig. 3). Variation of the back-flush time (connection time of C1 and C2) between 2 and 4 min

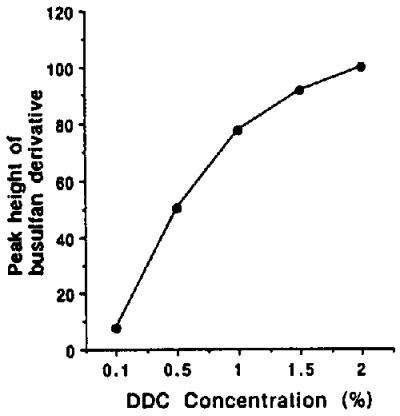

Fig. 2. Effect of the concentration of DDC aqueous solution [MP(A)] on the formation of the busulfan derivative.

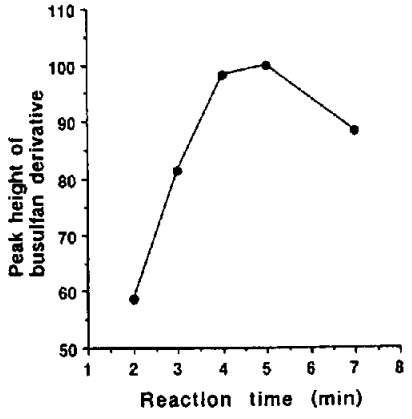

Fig. 3. Effect of the reaction time on C1 on the formation of the busulfan derivative.

had no effect on the peak height of the derivative on C2, and 3 min was selected (data not shown).

As a clean-up method for serum prior to HPLC analysis, liquid–liquid extraction was adopted. As the extraction solvent, ethyl acetate, diethyl ether and diethyl ether–dichloromethane (7:3, v/v) were examined, and the last one offered the best extraction efficiency.

Although reproducible derivatization was achieved with the above-described conditions, the sensitive determination of busulfan in serum was clearly impossible owing to interferences, mostly derived from DDC (Fig. 4). To obtain further purification, a heart-cutting technique was adopted following the derivatization on C1. The eluate fraction containing the busulfan derivative on C2 was further analysed on C3 by column switching. This procedure led to a chromatogram free from interferences at the retention time of the busulfan derivative (Fig. 5) and consequently allowed the sensitive determination of busulfan in human serum. A clean-up column

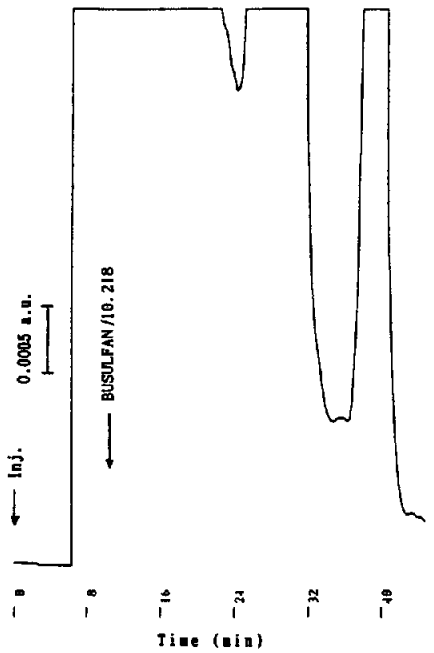

Fig. 4. Typical chromatogram of drug-free serum obtained with C1 and C2. The arrow indicates the retention time of the busulfan derivative.

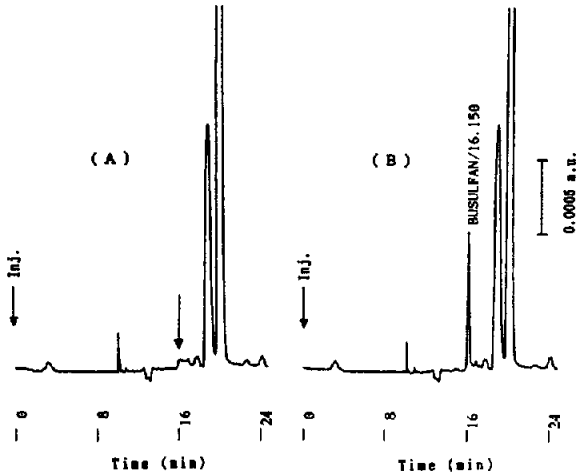

Fig. 5. Typical chromatograms of (A) drug-free serum and (B) serum spiked with busulfan (100 ng/ml) obtained with C1, C2 and C3. The arrow indicates the retention time of the busulfan derivative.

(C0) inserted between P(A) and the autoinjector was effective in eliminating the impurities in DDC solution [MP(A)]. The flow of MP(A) was stopped after the reaction (from 5.1 to 32 min) to decrease the amount of impurities retained on C0. This column was replaced every day.

A calibration graph was obtained by analysing spiked serum samples over the concentration range 10–2000 ng/ml. The weighted (1/concentration) least-squares regression fit showed good linearity, passing through the origin [slope = 0.357 ± 0.08 (S.D.), intercept = 0.642, five determinations for each five data points, correlation coefficient = 0.9999]. The recoveries from serum were 93–100% over the concentration range 10–2000 ng/ml. Accuracy and precision data are presented in Table 1. The lower limit of quantitation was 10 ng/ml. The method was sensitive enough for measuring busulfan concentrations in human serum in high-dose treatment.

In conclusion, the combination of on-line derivatization with DDC and a heart-cutting technique permitted the development of an HPLC method for the determination of busulfan in human serum. This automated and therefore reproducible derivatization method would be suitable for routine analysis.

Table 1
Accuracy and precision data for busulfan in human serum

Added concentration (ng/ml)	Mean found concentration (ng/ml)[a]	
	Intra-assay ($n = 5$)	Inter-assay ($n = 3$)
2000	2008.8 (2.5)	1998.3 (0.5)
500	489.5 (1.1)	500.3 (2.0)
100	101.3 (0.2)	101.6 (1.0)
20	20.7 (2.3)	20.0 (4.0)
10	9.7 (5.0)	9.8 (4.2)

[a] Values in parentheses are coefficients of variation (%).

Acknowledgements

The authors are grateful to Ryuichi Ohta (Takeda Chemical Industries) for the helpful discussions during the course of this study and Masumi Kataoka (Takeda Chemical Industries) for valuable information on the precolumn derivatization reaction with DDC.

References

[1] L. Embree, R.B. Burns, J.R. Heggie, G.L. Phillips, D.E. Reece, J.J. Spinelli, D.O. Hartley, N.J. Hudon and J.H. Goldie, *Cancer Chemother, Pharmacol.*, 32 (1993) 137.
[2] H. Ehrsson and M. Hassan, *J. Pharm. Sci.*, 72 (1983) 1203.
[3] G. Vassal, M. Re and A. Gouyette, *J. Chromatogr.*, 428 (1988) 357.
[4] M. Hassan and H. Ehrsson, *J. Chromatogr.*, 277 (1983) 374.
[5] T.-L. Chen, L.B. Grochow, L.A. Hurowitz and R.B. Brundrett, *J. Chromatogr.*, 425 (1988) 303.
[6] S. Pichini, I. Altieri, A. Bacosi, S.D. Carlo and P. Zuccaro, *J. Chromatogr.*, 581 (1992) 143.
[7] J. Blanz, C. Rosenfeld, B. Proksch and G. Ehninger, *J. Chromatogr.*, 532 (1990) 429.
[8] W.D. Henner, E.A. Furlong, M.D. Flaherty and T.C. Shea, *J. Chromatogr.*, 416 (1987) 426.
[9] J.J. MacKichan and T.P. Bechtel, *J. Chromatogr.*, 532 (1990) 424.
[10] A.G. Kazemifard and D.J. Morgan, *J. Chromatogr.*, 528 (1990) 274.