ELSEVIER

Journal of Chromatography B, 742 (2000) 427–432

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Rapid and sensitive high-performance liquid chromatographic method for busulfan assay in plasma

Nathalie Bleyzac*, Philippe Barou, Gilles Aulagner

Pharmacy Department, Debrousse Hospital, 29 rue Sœur Bouvier, 69005 Lyon, France

Received 1 November 1999; received in revised form 17 February 2000; accepted 1 March 2000

Abstract

A reversed-phase liquid chromatographic method with ultraviolet detection has been developed to determine busulfan concentrations in plasma of children undergoing bone marrow transplantation. Plasma samples (200 μl) containing busulfan and 1,6-bis(methanesulfonyloxy)hexane as an internal standard were prepared by a simple derivatization method with diethyldithiocarbamate followed by extraction with ethyl acetate and solid-phase purification on C_8 columns conditioned with methanol and water and eluted with acetonitrile (recovery 99%). Chromatography was accomplished using a Hypersil octadecylsilyl column (10 cm×4.6 mm I.D.) and a mobile phase of acetonitrile, tetrahydrofuran and distilled water (65:5:30, v/v). The limit of detection was 25 ng/ml (signal-to-noise ratio of 5). Calibration curves were linear up to 25 000 ng/ml. Intra-day and inter-day coefficients of variation of the assay were ≤5%. This method was used to analyse busulfan plasma concentrations after oral administration within the framework of therapeutic drug monitoring and pharmacokinetic studies in children.

Keywords: Busulfan

1. Introduction

Busulfan (1,4-butanediol dimethanesulphonate) is a bifunctional alkylating agent used at high doses (16 mg/kg/day divided in four doses, for four days) as a preparative regimen before bone marrow transplantation (BMT). Inter-patient pharmacokinetic variability of busulfan is wide in children [1,2] and does not always allow a safe and efficient use of this drug during BMT preparative regimens [1,2]. Therefore, we decided to develop a busulfan assay in plasma to apply therapeutic drug monitoring (TDM) in all of the patients submitted to BMT preparative regimens containing busulfan. The assay requirements were of a sufficient sensitivity and precision to allow pharmacokinetic studies and a short analysis time to get the plasma concentration results rapidly, in order to modify busulfan dosage regimens if necessary as early as the third daily dose.

A lot of methods have already been developed for the determination of busulfan in plasma. Gas chromatographic (GC) techniques coupled with mass spectrometry [3,4] are known to be the most sensitive and specific methods but they are time-consuming and difficult to use in routine clinical practice. GC with electron-capture detection [5–7] is sensitive, but analysis times are too long or sample

*Corresponding author. Tel: +33-472-38-5776; fax: +33-472-38-5515.

E-mail address: bleyzac@caramail.com (N. Bleyzac)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00167-5

volume needed is too important for paediatric patients.

High-performance liquid chromatography (HPLC) [1,8–16] is the most frequently used method. However, published assays require costly equipments when UV detection is not used [8,13] or are not sensitive, specific or precise enough [9,11,15,16] or do not give a result rapidly enough for a daily busulfan dose adjustment [1,9–16].

Therefore, we describe a HPLC assay of busulfan combining all the advantages missing in the methods previously described, that is to say a good precision and sensitivity, a small volume of plasma sample (200 μl), and a very short analysis time due to a simplified sample treatment method involving a one-step derivatization procedure and a shortened evaporation step.

2. Experimental

2.1. Materials

Busulfan was obtained from Techni Pharma (Monaco), methanol, acetonitrile and ethyl acetate (HPLC grade) were purchased from Merk (Chelles, France), tetrahydrofuran (HPLC grade) from Prolabo (Fontenay sous Bois, France), sterile distilled water Versol® from Aguettant (Lyon, France), diethyldithiocarbamate, methylene chloride, methanesulfonyl chloride, and pyridine from Sigma (Saint Quentin Fallavier, France) and 1,6-hexanediol from Fluka (Saint Quentin Fallavier, France). Drug-free control serum Lyotrol N® was purchased from Biomérieux (Marcy l'Etoile, France).

2.2. HPLC apparatus

Liquid chromatographic apparatus was composed of a Shimadzu LC-6A pump, a Shimadzu C-R3A integrator/recorder, a Shimadzu SPD-6A UV/Vis detector, and a Shimadzu SIL-9A autoinjector from Touzart and Matignon (Courtaboeuf, France). The column used was a C_{18} Hypersil ODS column (3 μm particle size, 10 cm×4.6 mm, from Touzart and Matignon). No pre-column was employed.

2.3. Chromatographic conditions

The separation of the busulfan and internal standard derivatives was achieved with an isocratic solvent delivery system at a flow-rate of 1.2 ml/min at room temperature. The mobile phase was constituted of acetonitrile, tetrahydrofurane and distilled water (65:5:30, v/v) and was degassed daily by using ultrasonics. Detection was performed at 280 nm.

2.4. Standards

Busulfan plasma standards were prepared by the following procedure: aliquots of drug-free serum (Lyotrol N, Biomérieux) were spiked with a 100 mg/l busulfan stock solution to obtain four levels, 200, 500, 1000 and 2000 ng/ml. Standards were prepared daily in propylene tubes.

2.5. Synthesis of internal standard

The 1,6-bis-(methanesulfonyloxy)hexane was synthesized using a method derived from those already published [6,15]. Forty ml of a 625 g/l methanesulfonyl chloride solution in methylene chloride were slowly added to 40 ml of a pyridine (435 g/l) and 1,6-hexanediol (365 g/l) solution in methylene chloride, while stirring at 0°C. The mixture was mixed at 25°C for 1 h using a magnetic agitator. The 1,6-bis(methanesulfonyloxy)hexane sample was then precipitated and crystallized with 2×40 ml of distilled water in a decantation balloon. The compound was collected as a powder after filtration of the aqueous phase. The powder was then dried at 55°C under nitrogen. The synthesized compound was checked for purity by the demonstration of a single peak by HPLC. The internal standard was kept at −20°C as a 100 mg/l solution in methanol. The frozen solution was stable for one year.

2.6. Blood sample collection

Blood was collected on heparine by venopuncture. Samples were immediately placed in an ice bath and centrifuged without delay at 4°C. Plasma was separated in polypropylene tubes and immediately assayed or stored at −20°C until it could be analysed.

Busulfan plasma samples were stable for up to six months at −20°C [17].

2.7. Sample treatment

A 200 μl sample of plasma was added to 30 μl of a 5 mg/l internal standard solution in plasma and deproteinized with 200 μl of acetonitrile. The mixture was mixed during 20 s exactly using a Top Mix 94323 vortex (Bioblock, Isle d'Abeau, France). Centrifugation before derivatization proved to be unnecessary for sample treatment improvement and additionally time-consuming and was not carried out at this step.

A simple derivative procedure was developed, using only diethyldithiocarbamate (DEDC). Derivatization took place between the deproteinization and extraction procedure. A 200 μl sample of a 50 g/l DEDC solution in water was added to the mixture of plasma and acetonitrile and was mixed for 5 s. The derivatives were immediately extracted with 200 μl of ethyl acetate, while mixing for exactly 1 min. The mixture was centrifuged at 2000 *g* and at 4°C for 15 min. The upper phase was transferred into borosilicated glass tubes and was evaporated to dryness at 55°C under nitrogen (5 min). The dry extract was solubilized in 100 μl of methanol. A 600 μl sample of water was added to enhance the mixture polarity in order to optimize the further solid-phase extraction.

2.8. Solid phase extraction

The above solution (700 μl) was purified on 1 ml (100 mg) Sep-Pak C8 125 Å solid extraction cartridges (Waters, St. Quentin-Yvelines, France), by using a vacuum apparatus (Visiprep®, Supelco, St Quentin Fallavier, France). Cartridges were conditioned with 1 ml of methanol followed by 1 ml of water. Each sample was slowly passed through the cartridge without delay (to avoid getting the phase dry) and exceeding no more than 1 ml/min. Cartridges were then washed with 1 ml of methanol–water (50:50, v/v) and dried. Derivatized compounds were eluted with 300 μl of acetonitrile. An aliquot of 30 μl of the eluate was injected into the liquid chromatograph.

3. Results

3.1. Chromatographic separation

Retention times of busulfan derivative and internal standard derivative were respectively 5.6 and 10.1 min (C.V. inter-day both ≤2%), corresponding to k' of 9.5 and 17.7. The drug-free plasma sample did not show any peak that would interfere with the analytes. Fig. 1 shows chromatograms of blank and spiked plasma. The resolution of the derivatives of busulfan and the internal standard from the components found

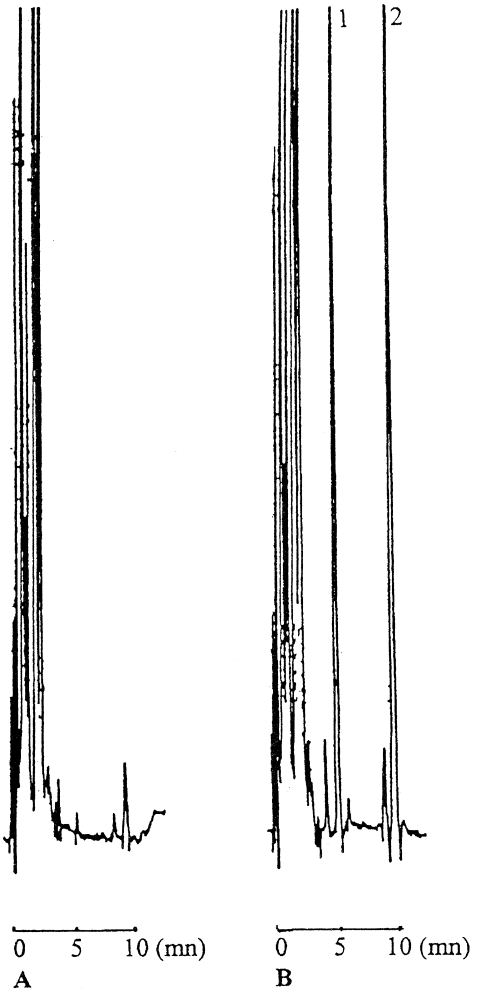

Fig. 1. Chromatograms of a blank plasma (A) and a plasma sample spiked with 1 μ/ml of busulfan (B). Peaks: 1=busulfan; 2=internal standard.

to elute just before 5 min and 10 min was more than 2.5, allowing a quite correct separation. The same results were obtained with blank plasma analysed at different day and with plasma samples from patients before bone marrow conditioning with busulfan. All the drugs able to be concomitantly administered with busulfan during bone marrow transplantation conditioning were tested for specificity of the assay. Drug-free plasma samples were spiked separately with an appropriate concentration of each drug (therapeutic concentration) and were treated exactly the same as described above. Drugs (and concentrations assayed) were aciclovir (100 mg/l), amikacine (40 mg/l), chlorpromazine (50 mg/l), clonazepam (10 mg/l), furosemide (100 mg/l), methotrexate (10 μmol/l) omeprazole (40 mg/l), ranitidine (250 mg/l), tienamycine (50 mg/l), vancomycine (50 mg/l). None of these compounds were eluted in the chromatographic system or detected at the wavelength used.

3.2. Quantitative analysis

Quantification was performed according to the internal standard method and by plotting the peak height ratio against known concentrations of spiked plasma standards (using a least-squares linear regression). Drug-free plasma samples ($n=10$) spiked with busulfan (1 mg/ml) were treated according to the procedure described above and were compared with busulfan in acetonitrile samples (1 mg/ml) treated in the same conditions. Both the busulfan and internal standard derivatives were respectively 99 ± 2 and $98\pm2\%$ ($n=10$) recovered from the sample treatment procedure. The limit of detection for busulfan in plasma was 25 ng/ml, based on a signal-to-noise ratio of 5. The limit of quantification was 50 ng/ml (signal-to-noise ratio of 10). Linearity was perfect for busulfan plasma concentrations ranging from 25 to 25 000 ng/ml ($r=0.999$, $P<0.001$). The upper limit is largely above the current plasma concentrations found in humans.

3.3. Accuracy and precision

The intra-day variability of the assay was tested on ten samples of two different concentrations of busulfan in plasma, all prepared on the same day. These samples were also used to test the assay accuracy. The inter-day variability of the assay was tested for two different concentrations of busulfan in plasma, for ten consecutive days (3 samples per day) (Table 1). The assay was accurate as no significant differences were found between the concentrations tested and target values (Student, $P=0.93$ and 0.92 for 200 and 2000 ng/ml respectively).

Table 1
Accuracy and precision of the busulfan assay

Assay	Concentration (ng/ml)	*n*	Mean measured concentration (ng/ml)	C.V. (%)
Intra-day	200	10	202	3.8
	2000	10	1980	2.9
Inter-day	200	10	199	5.3
	2000	10	2019	4.8

3.4. Pharmacokinetic studies

The chromatographic assay described here has been applied to pharmacokinetic dose adjustment of busulfan in children during a bone marrow transplantation preparative regimen [18,19]. Busulfan was administered orally for four days (1 mg/kg every 6 h). A test-dose of 0.5 mg/kg was given one week before starting preparative regimen and allowed us to determine the best dosage regimen to reach a target AUC (area under the plasma concentration curve over time) ranging between 4 and 6 (μg/ml)h [17]. Busulfan pharmacokinetic parameters are determined for each patient using Bayesian techniques (USC*PACK software, [20]). Fig. 2 shows a typical patient pharmacokinetic profile. Therapeutic drug monitoring was performed during the four days of the conditioning regimen (two measures of busulfan plasma concentration per day, 1.5 and 5 h after drug administration). A Bayesian adaptive control of busulfan dosage regimen was performed daily in order to maintain plasma concentrations and AUC in the target ranges. Pharmacokinetic individualisation of dosage regimens improved the use of busulfan, especially by decreasing the number of serious side effects such as veno-occlusive disease [20].

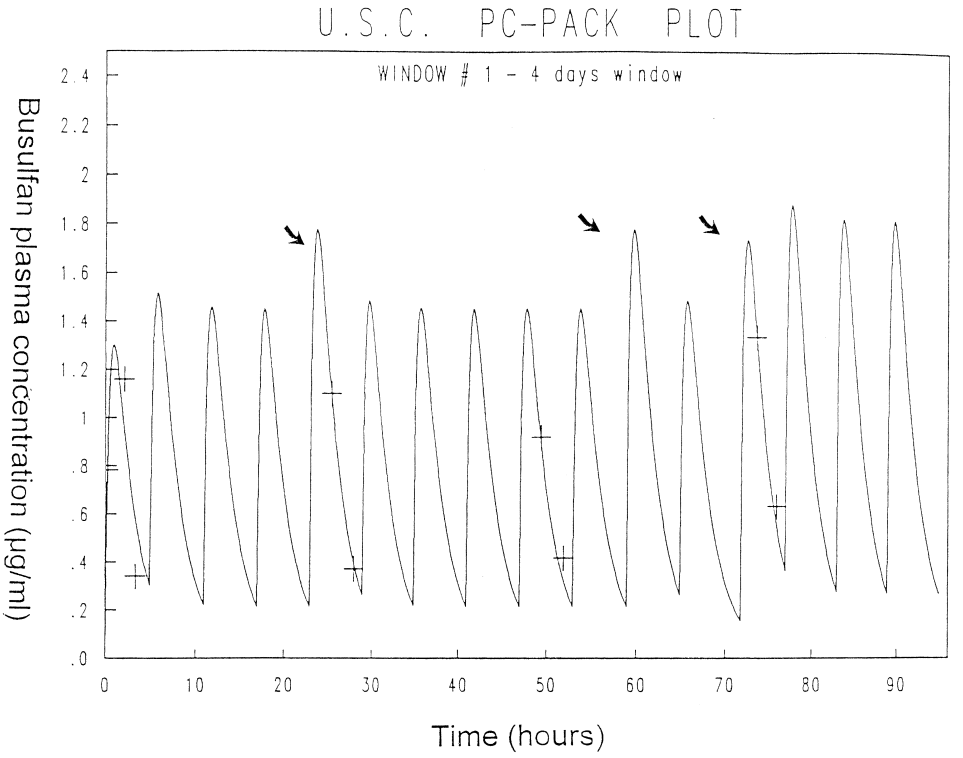

Fig. 2. Typical four-day pharmacokinetic profile of busulfan in a child before bone marrow transplantation. Initial dosage was 40 mg/6 h. Dosage was adjusted several times to 50 mg (↘), according to busulfan plasma levels (+) to achieve target AUC per dose (5 $\mu g \cdot h \cdot ml^{-1}$).

4. Discussion

The above described method is the most rapid HPLC assay for busulfan determination in plasma. Sample treatment and chromatographic analysis require only 32 and 10 min respectively. In fact, two steps (derivatization and liquid extraction) were considerably simplified with regards to others methods [9–16]. Only one reagent was employed for each step and all reagent volumes were notably decreased to optimize the evaporation step. Solid phase extraction duration could not be shortened. Nevertheless, this step was necessary to purify the evaporation residue. In addition, our method needs only simple chromatographic apparatus (isocratic elution, UV detection), available in all hospital laboratories. Moreover, despite a simplified sample treatment, the assay remains quite sensitive. These conditions make it quite suitable for high-return therapeutic drug monitoring, when there is a need to get results rapidly for daily or twice-daily dose adjustment. Also, it is particularly easy to perform since no special equipment is needed. Indeed, only three existing methods present a detection limit as low as that of our method [8–10] but the analysis time is much longer (3–4 h).

Existing HPLC methods do not present simultaneously both the characteristics required for paediatric patients, such as a small volume of plasma sample, and a high precision and high sensitivity to satisfy pharmacokinetic studies. Indeed, only two published HPLC methods use a 200 µl plasma sample [15,16] but they are not sensitive enough. Moreover, the precision is not sufficient with between-day C.V. above 14% [15] or the linearity range is not large enough [16]. The assay method described above meets all the requirements for pharmacokinetic studies [21].

Most of the existing HPLC methods used methanol for plasma deproteinization. We preferred to use acetonitrile as methanol may lead to hydrolysis of the derivatives (Laboratory of Organic Chemistry, Lyon I University, personal communication). Moreover, particular care was taken in treating the blood samples, limiting the loss of busulfan. Busulfan is known to be adsorbed on glass, which is the reason why plasma is always treated in polypropylene or borosilicated glass tubes. All these precautions contribute to enhancing reproducibility and sensitivity.

We chose 1,6-bis-(methanesulfonyloxy)hexane as an internal standard because its chemical structure is close to that of busulfan. In fact, this compound presents only an additional ethylene group with regards to busulfan. Consequently, it undergoes the same derivatization process, allowing the control of the reaction efficiency. Behaviour during extraction is also the same as that of busulfan. Moreover, the retention time of the derivatized compound is close to that of busulfan. Therefore, the analysis duration was not penalized.

In summary, this method provides several advantages over all existing methods. Therapeutic monitoring of busulfan is widely recommended since overdoses can cause severe side effects. This simple and easy to perform method could allow to increase the number of monitored patients.

References

[1] L.B. Grochow, Sem. Oncol. 20 (1993) 18.
[2] G. Vassal, Anticancer Res. 14 (1994) 2363.
[3] H. Ehrsson, M. Hassan, J. Pharm. Sci. 72 (1983) 1203.
[4] G. Vassal, M. Re, A. Gouyette, J. Chromatogr. 428 (1988) 357.
[5] R.B. Burns, J.R. Heggie, L. Embree, J. Pharm. Biomed. Anal. 13 (1995) 1073.
[6] T.L. Chen, L.B. Grochow, L.A. Hurowitz, R.B. Brundrett, J. Chromatogr. 425 (1988) 303.
[7] M. Hassan, H. Ehrsson, J. Chromatogr. 277 (1983) 374.
[8] J. Blanz, C. Rosenfeld, B. Proksch, G. Ehninger, J. Chromatogr. 532 (1990) 429.
[9] I.K.I. Funakoshi, K. Yamashita, W.F. Chao, M. Yamaguchi, T. Yashiki, J. Chromatogr. 660 (1994) 200.
[10] J.R. Heggie, M. Wu, R.B. Burns, H.C. Fung, G. Knight, M.J. Barnett, J.J. Spinelli, L. Embree, J. Chomatogr. 692 (1997) 437.
[11] W.D. Henner, E.A. Furlong, M.D. Flaherty, T.C. Shea, J. Chromatogr. 416 (1987) 426.
[12] J.J. MacKichan, T.P. Bechtel, J. Chromatogr. 532 (1990) 424.
[13] S. Pichini, I. Altieri, A. Bacosi, S. Di Carlo, P. Zuccaro, J. Chromatogr. 581 (1992) 143.
[14] N. Rifai, M. Sakamoto, M. Lafi, E. Guinan, Ther. Drug Monit. 19 (1997) 169.
[15] D.S. Chow, H.P. Bhagwatwar, S. Phadungpojna, B.S. Andersen, J. Chromatogr. B 704 (1997) 277.
[16] M.H. Quernin, B. Poonkuzhali, Y. Medard, D. Dennison, A. Srivastava, R. Krishnamoorthy, M. Chandy, E. Jacqz-Aigrain, J. Chromatogr. B 721 (1999) 147.
[17] L.B. Grochow, R.J. Jones, R.B. Brundett, H.G. Braine, T.L. Chen, R. Saral, G.W. Santos, O.M. Colvin, Cancer Chemother. Pharmacol. 25 (1989) 55.
[18] N. Bleyzac, P. Magron, G. Souillet, P. Barou, G. Sahyoun, P. Maire, G. Aulagner, Hematol. Cell Ther. 40 (1998) 205.
[19] N. Bleyzac, P. Magron, P. Barou, G. Souillet, G. Aulagner, Bone Marrow Transplant. 23 (1999) S71.
[20] Laboratory of Applied Pharmacokinetics, School of Medicine, Los Angeles, version 10-7.
[21] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokin. 16 (1991) 249.