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Determination of busulfan in human plasma using high-performance liquid chromatography with pre-column derivatization and fluorescence detection

José-Esteban Peris[a,*], José-Antonio Latorre[a], Victoria Castel[b], Amparo Verdeguer[b], Sara Esteve[a], Francisca Torres-Molina[a]

[a]*Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Valencia, Avda. V. Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*

[b]*Servicio de Pediatría Oncológica, Hospital Infantil La Fe, Valencia, Spain*

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Abstract

A rapid, sensitive and reproducible high-performance liquid chromatographic assay for busulfan in human plasma was developed. After extraction of plasma samples with acetonitrile and methylene chloride, busulfan and the internal standard [1,5-bis(methanesulfonyloxy)pentane] were derivatized with 8-mercaptoquinoline to yield fluorescent compounds which were detected with a fluorescence detector equipped with filters of 360 nm (excitation) and 425 nm (emission). Calibration graphs showed a linear correlation ($r>0.9990$) over the concentration range of 20–2000 ng/ml. The recovery of busulfan from plasma standards was 70±5%. The detection and quantification limits for busulfan in plasma samples were established at 9 ng/ml and 20 ng/ml, respectively. The intra- and inter-assay variations were lower than 8% and 10%, respectively. The applicability of the method was verified by analyzing the plasma concentrations of busulfan in a patient to whom it was administered orally on two different days.

Keywords: Busulfan

1. Introduction

Busulfan (1,4-butanediol dimethanesulfonate) is a bifunctional alkylating agent which is used for the treatment of chronic myelogenous leukemia and is administered at dosages of 0.065 to 0.1 mg/kg daily [1]. Busulfan is also used at high doses (4 mg/kg daily) in combination with cyclophosphamide as a preparative regimen for bone marrow transplantation (BMT) procedures. The adverse effects of busulfan includes hematological toxicity (severe leukopenia, anemia and thrombocytopenia), hyperuricemia, bronchopulmonary dysplasia, hepatic dysfunction and cholestatic jaundice, etc. [1]. Furthermore, when busulfan is administered at high doses a high incidence of the veno-occlusive disease (VOD) of the liver has been reported; it arises in approximately 20 to 40% of the patients undergoing BMT [2–8]. About 50% of the patients that develop VOD die. Although a clear causal relationship between VOD and busulfan has not yet been established, several authors have observed higher plasma concentrations

*Corresponding author.

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of busulfan in patients who developed VOD and have addressed the convenience of busulfan plasma monitoring in order to individualize the dose of this drug and decrease the incidence of VOD [7,9–14].

Several analytical methods have been developed to determine busulfan in plasma samples. These include gas chromatography–mass spectrometry (GC–MS) [15], GC with electron-capture detection [16,17] and high-performance liquid chromatography (HPLC) with UV detection [9,18,19] or selected ion monitoring (SIM) [20]. Whereas GC-based methods seem to achieve a good sensitivity and show lack of interfering peaks, methods based on HPLC with UV detection show poor sensitivity and/or the presence of interfering peaks [9,18,21].

The methanesulfonate moieties of busulfan can undergo nucleophilic substitution by electrophilic molecules, such as iodide or thiol compounds. Therefore, we decided to try using 8-mercaptoquinoline as a fluorogenic tag for busulfan. This paper describes a simple, reliable method for assaying busulfan by HPLC using pre-column derivatization and fluorescence detection which has been used to analyze plasma concentrations of busulfan in a patient.

2. Experimental

2.1. Materials

Busulfan, 8-mercaptoquinoline and tri-*n*-butylphosphine were obtained from Sigma (St. Louis, MO, USA). Methanesulfonyl chloride was from Aldrich (Steinheim, Germany) and 1,5-pentanediol was from Fluka (Buchs, Switzerland). Pyridine and methylene chloride were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical or HPLC grade.

1,5-Bis(methanesulfonyloxy)pentane was used as an internal standard and was synthesized as follows [16]: a solution of methanesulfonyl chloride (2.52 g, 0.022 mol) in methylene chloride (4 ml) was added slowly to a solution of 1,5-pentanediol (1.04 g, 0.01 mol), pyridine (1.74 g, 0.022 mol) and methylene chloride (4 ml) while stirring at 0°C. The mixture was stirred at 25°C for 1 h and extracted four times with water (7 ml). Methylene chloride was evaporated at 55 to 65°C to obtain a viscous liquid to which 5 ml of ethanol was added. The mixture was then shaken and placed in a refrigerator (approximately −10°C) to obtain the separation of both phases. The ethanol phase was discarded and the product was washed again with 5 ml of ethanol. After heating at 70°C to eliminate the ethanol residue, 1,5-bis(methanesulfonyloxy)pentane was obtained as a viscous liquid and was used without any additional purification. Nuclear magnetic resonance (NMR) ($CDCl_3$), δ 4.2 ($-OCH_2$, triplet), δ 3.0 ($-CH_3$, singlet), δ 1.8 ($-CH_2CH_2$, multiplet), δ 1.5 ($-CH_2$, multiplet).

2.2. Stock solutions

Busulfan (100 mg) was accurately weighed and dissolved in 10 ml of dimethylformamide. Serial dilutions in the range of 2–200 μg/ml were made in dimethylformamide, and standards in plasma were prepared by adding 10 μl of these solutions to 1 ml of plasma (20–2000 ng/ml). A solution of the internal standard in dimethylformamide was obtained by serial dilutions to a final dilution of 10 μg/ml. Busulfan and internal standard solutions were stored at −30°C and were stable for at least two years. The other reagent solutions, 8-mercaptoquinoline in dimethylformamide (1 mg/ml) and tri-*n*-butylphosphine in dimethylformamide (1%, v/v), were also stored at −30°C and prepared monthly.

2.3. Extraction and derivatization of plasma samples

Plasma (1 ml) and 20 μl of the internal standard solution (10 μg/ml) were pipetted into a tube and 1 ml acetonitrile was added. The mixture was vortex-mixed for 30 s and centrifuged for 5 min (1000 *g*). A 1.5-ml volume of supernatant was removed to a clean screw-capped glass tube and 3 ml methylene chloride was added. After capping the tube, the mixture was shaken for 1 min by hand followed by 15 min in a type DSG-301 Heidolph shaker. It was then centrifuged for 5 min (1000 *g*). The upper aqueous phase was removed and the organic phase was dried under nitrogen at 50°C. Then 10 μl of tri-*n*-butylphosphine solution, 100 μl of 8-mercaptoquinoline solution and 10 μl of 0.1 *M* NaOH were

added to the residue and, after capping the tube, the mixture was heated at 80°C for 1 h. A volume of 20 μl was injected into the HPLC system.

2.4. Instrumentation and conditions

A Perkin-Elmer Model Series-10 pump was used to deliver a mixture of acetate buffer (100 m*M*, pH 5)–acetonitrile (45:50, v/v) at 1 ml/min through a Nova Pak C_{18} cartridge of 15 cm×3.9 mm I.D. (Waters, Milford, MA, USA). Detection was performed with a Waters Model 420-AC fluorescence detector equipped with filters of 360 nm (excitation) and 425 nm (emission). Peaks were recorded with a Perkin-Elmer Model LCI-100 integrator.

2.5. Calibration curves

Calibration standards for busulfan, covering the range 20–2000 ng/ml, were prepared in 1 ml of drug-free plasma and subjected to the extraction and derivation procedure indicated above. The calibration curve was obtained by plotting the peak-height ratio of busulfan/internal standard versus the nominal concentration of busulfan. The slope and intercept of the calibration line was determined by linear regression using the least-squares method.

2.6. Method validation

Four quality control samples (20, 100, 400 and 2000 ng/ml) were prepared in plasma and analyzed on the same day ($n=6$) to establish the intra-day precision. The assay was repeated ($n=5$) over an eight-month period to establish the inter-day precision. The intra- and inter-day precision were expressed as the relative standard deviation (RSD) of the observed concentrations.

The accuracy of the method was evaluated by comparing the mean concentration observed and the theoretical value of busulfan in each quality control sample. The accuracy was expressed as percent relative error.

To calculate the recovery, spiked plasma samples (100, 500, 1000 and 2000 ng/ml) were analyzed and the peak-heights were compared with those of corresponding non-extracted standards of busulfan prepared in dimethylformamide and subjected to the same derivatization procedure.

The limit of detection (LOD) was determined as the analyte concentration in plasma samples giving rise to a signal-to-noise ratio of 3. The limit of quantification (LOQ) was set at the lowest standard concentration on the calibration curve.

2.7. Application of the method

To test the applicability of the described method for pharmacokinetic studies, plasma samples of a patient receiving busulfan orally were analyzed. Blood samples were withdrawn from a forearm vein 0, 20, 30, 60, 120, 180, 240 and 360 min after administration of 1 mg/kg of busulfan on two consecutive days. Blood was placed in heparinized tubes and plasma was immediately separated and frozen at −20°C until analysis.

3. Results and discussion

3.1. Analytical method

The reaction of busulfan with 8-mercaptoquinoline in an alkaline medium gave a highly fluorescent derivative, with maximum excitation and emission wavelengths of 350 and 430 nm, respectively. Therefore, a excitation filter of 360 nm and a emission filter cut-off of 425 nm were selected for the detection in the chromatographic system. The chemical structures of busulfan, internal standard and derivatization reagent (8-mercaptoquinoline), as well as of the proposed corresponding derivatives are shown in Fig. 1. The reaction of busulfan and the internal standard with 8-mercaptoquinoline was fast at 80°C, as shown in Fig. 2. An alkaline medium was essential for the reaction, as was the addition of tri-*n*-butylphosphine to avoid the oxidation of the reagent. The peak-height ratio of busulfan derivative to internal standard derivative (Fig. 2B) was practically constant after 30 min of reaction.

Chromatograms of blank plasma and plasma with busulfan are shown in Fig. 3, where it can be observed that there are no interfering peaks of endogenous plasma components and that peaks of

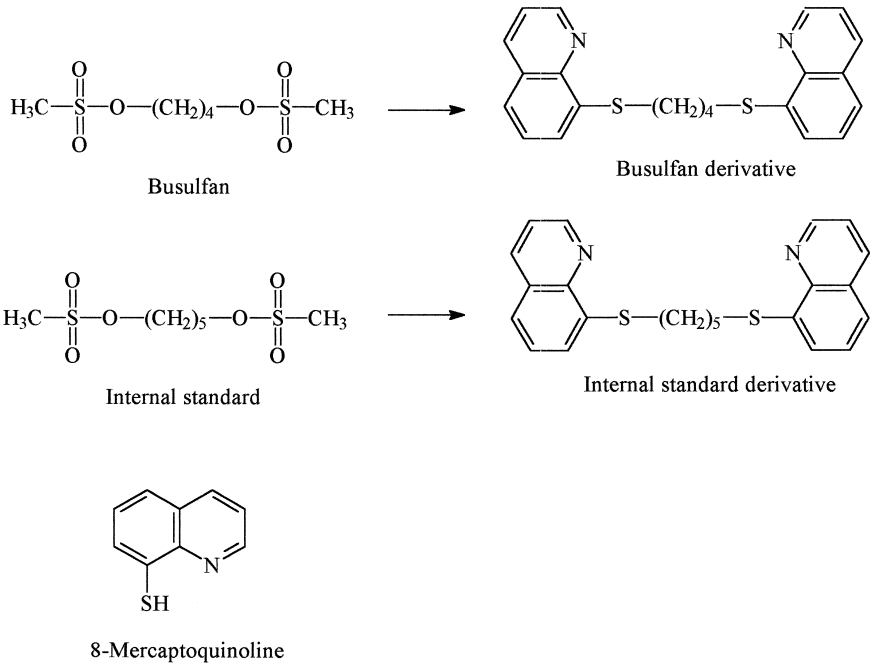

Fig. 1. Chemical structures of busulfan, internal standard, and their derivatives obtained after reaction with 8-mercaptoquinoline.

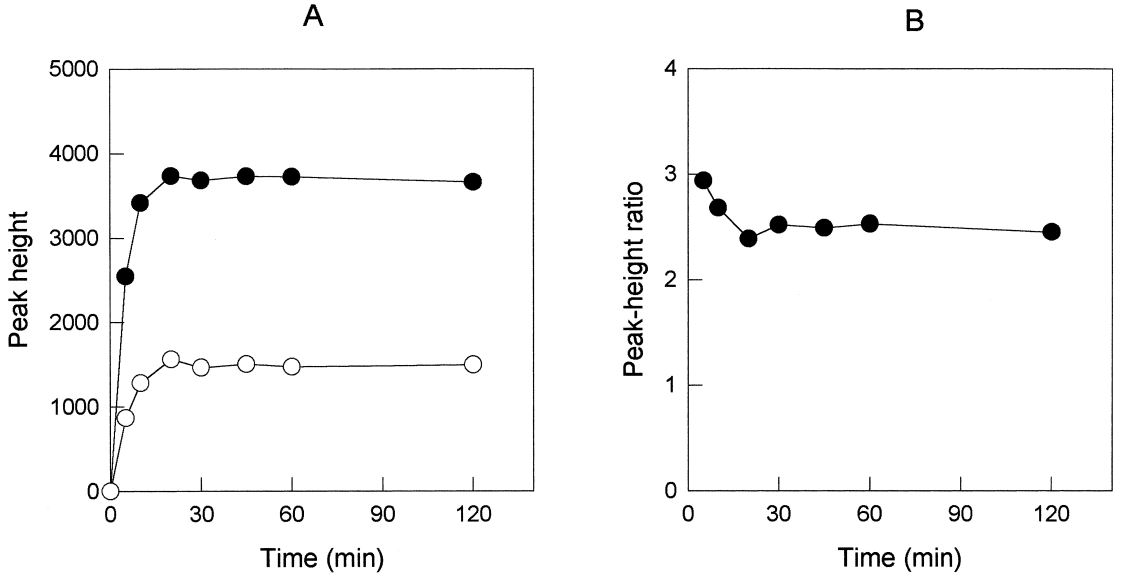

Fig. 2. (A) Peak-height of derivatives of busulfan (•) and internal standard (○) as a function of time of reaction in the conditions indicated in the text. (B) Peak-height ratio of busulfan derivative to internal standard derivative as a function of time of reaction.

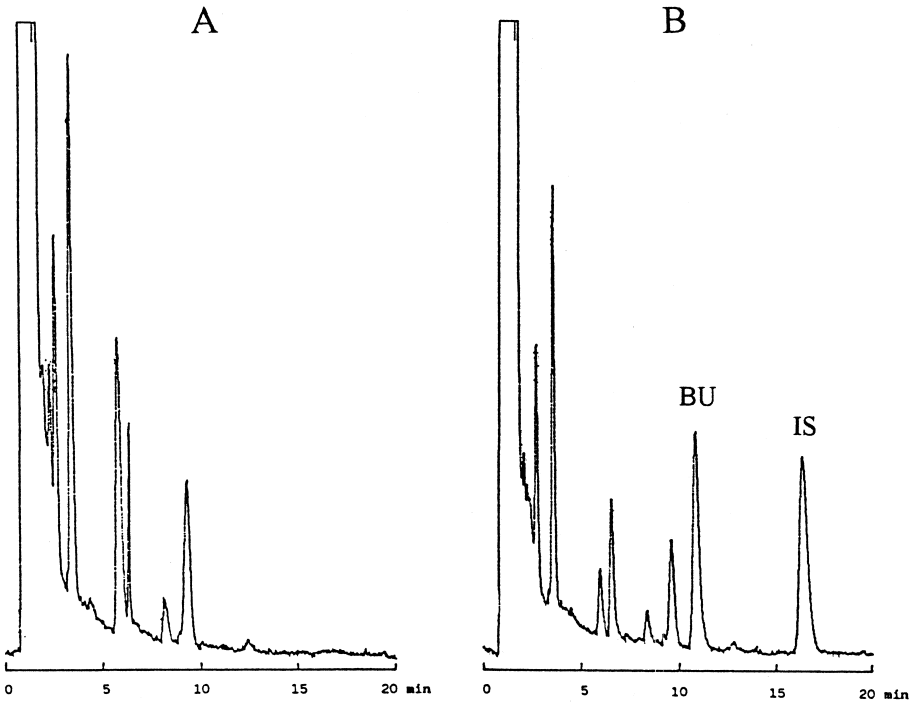

Fig. 3. (A) Chromatogram of blank plasma treated according to the method described in the text. (B) Chromatogram of plasma spiked with busulfan (BU) and internal standard (I.S.).

busulfan and internal standard derivatives are completely resolved.

Regression analysis of the calibration curves showed a linear relationship between the peak-height ratio of busulfan/internal standard and the busulfan concentration, with correlation coefficients higher than 0.9990 in all the curves assayed. The accuracy and precision of the assay are presented in Table 1. Accuracy, estimated as the deviation of the observed mean concentration from the actual concentration, was less than 5% for all the concentrations assayed. The intra- and inter-day RSDs were lower than 10% for all concentrations assayed. With this method, concentrations of busulfan in plasma as low as 20

Table 1
Precision and accuracy of busulfan assay in plasma

Nominal concentration (ng/ml)	Concentration found (ng/ml)	Accuracy (Found/nominal, %)	Precision (RSD, %)
Intra-assay (n=6)			
20	20.3±1.5	102	7.4
100	96.7±5.3	97	5.5
400	406.4±13.1	102	3.2
2000	2053.7±115.9	103	5.6
Inter-assay (n=5)			
20	20.9±2.0	104	9.5
100	94.9±8.0	95	8.4
400	401.1±28.0	100	7.0
2000	2029.3±65.2	101	3.2

ng/ml could be precisely quantified (LOQ) with a LOD of approximately 9 ng/ml, based on a signal-to-noise ratio of 3. The recovery in the 100 to 2000 ng/ml range was found to be 70±5%.

The busulfan molecule is devoid of any chromophoric system and therefore cannot be detected by the commonly used HPLC detectors (UV and fluorescence). To overcome this difficulty, pre-column derivation [9,18] and post-column photolysis [19] methods have been developed to permit UV detection of plasma samples containing busulfan. Pre-column derivation with sodium diethyldithiocarbamate to form the 252-nm UV-absorbing derivative 1,4-bis(diethyldithiocarbomyl)butane [18] gives chromatograms with potentially interfering peaks [21], and this limits the usefulness of this method for measuring plasma busulfan concentrations. A modification of this method has recently been published that seems to avoid the presence of interfering peaks in the chromatograms [22], but the molecular structure of the internal standard used in this method is very different from that of busulfan and does not react with the derivatizing agent. Pre-column derivatization with thiocresol and UV detection at 254 nm [9] gives also potentially interfering peaks with a detection limit of 0.4 μmol/l (98.5 ng/ml) when a volume of 2.5 ml of plasma is used. Pre-column derivatization of busulfan with sodium iodide leads to 1,4-diiodobutane, which can be chromatographed and detected after on-line post-column photolysis, since the iodine ions obtained by the photodissociation of the carbon–iodine bonds absorb 226-nm UV light [19]. The chromatograms obtained with this method do not present interfering peaks from plasma components and the LOD is low (20 ng/ml). However, this method requires a photochemical reactor (which was handmade by the authors), the peak due to the busulfan derivative is very wide, and although the sample treatment includes three extractive steps (the first with a C_8 cartridge, the second with *n*-heptane, and the third with 2-methoxyethanol), an adequate internal standard is not available.

The combination of HPLC–MS with SIM seems to avoid the presence of interfering peaks in the

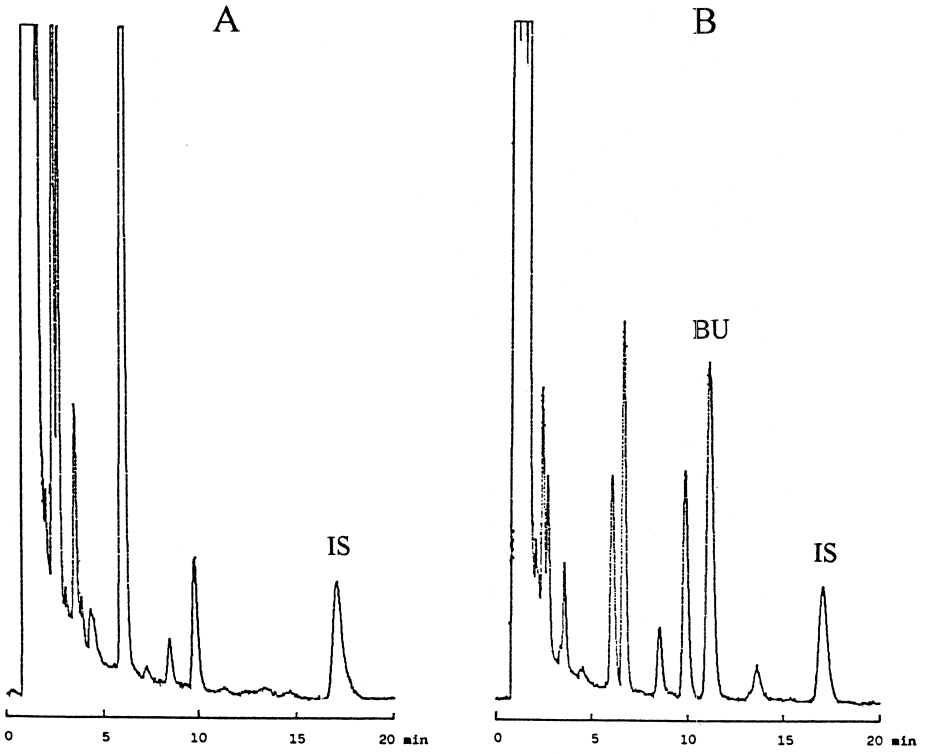

Fig. 4. (A) Chromatogram of a plasma sample obtained before administration of busulfan. (B) Chromatogram of a plasma sample obtained 6 h after the oral administration of 1 mg/kg of busulfan.

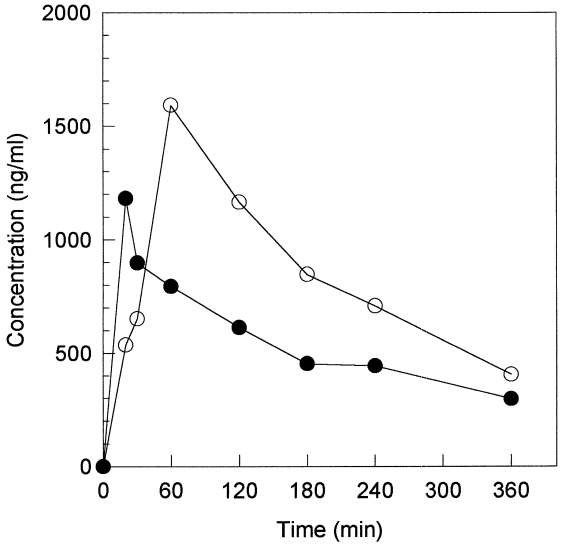

Fig. 5. Plasma concentrations versus time plot of busulfan in a patient after the oral administration of 1 mg/kg on two consecutive days: ● day 1; ○ day 2.

chromatogram [20], but the fact that this method is not very sensitive (detection limit: 100 ng/ml) and requires expensive equipment, restricts its use for routinely monitoring the plasma concentrations of busulfan in patients.

3.2. Applicability of the method

The analytical method was used to determine the plasma concentration of busulfan in a patient. In Fig. 4, chromatograms corresponding to plasma samples obtained before and after 6 h of drug administration are shown. There were no interfering plasma peaks and the drug was detected with a high signal 6 h after drug administration, which indicates that the method can be used to run pharmacokinetic studies with this drug. The plasma concentration–time profile of busulfan after oral administration to the same patient on two different days was very different (Fig. 5), which suggests a marked intra-patient variability in busulfan pharmacokinetics.

4. Conclusions

A liquid chromatographic method for quantifying busulfan in plasma samples has been developed and validated in human plasma. The assay is selective, precise, accurate and linear over the concentration range studied. Using 1 ml of plasma, concentrations of busulfan as low as 20 ng/ml could be precisely quantified, and the LOD was approximately 9 ng/ml. The method is simple and suitable for the determination of plasma busulfan in pharmacokinetic studies.

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