

# A novel method for quantification of sulfolane (a metabolite of busulfan) in plasma by gas chromatography–tandem mass spectrometry

François Versace · Chakradhara Rao S. Uppugunduri · Maja Krajinovic · Yves Théorêt · Fabienne Gumy-Pause · Patrice Mangin · Christian Staub · Marc Ansari

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**Abstract** The role of busulfan (Bu) metabolites in the adverse events seen during hematopoietic stem cell transplantation and in drug interactions is not explored. Lack of availability of established analytical methods limits our understanding in this area. The present work describes a novel gas chromatography–tandem mass spectrometric assay for the analysis of sulfolane (Su) in plasma of patients receiving high-dose Bu. Su and Bu were extracted from a single 100  $\mu$ L plasma sample by liquid–liquid extraction. Bu was separately derivatized with 2,3,5,6-tetrafluorothiophenol fluorinated agent. Mass spectrometric detection of the analytes was performed in the selected reaction monitoring mode on a triple quadrupole instrument after electronic impact ionization. Bu and Su were analyzed with separate chromatographic programs, lasting 5 min each. The assay for Su was found to be linear in the

concentration range of 20–400 ng/mL. The method has satisfactory sensitivity (lower limit of quantification, 20 ng/mL) and precision (relative standard deviation less than 15 %) for all the concentrations tested with a good trueness ( $100 \pm 5$  %). This method was applied to measure Su from pediatric patients with samples collected 4 h after dose 1 ( $n=46$ ), before dose 7 ( $n=56$ ), and after dose 9 ( $n=54$ ) infusions of Bu. Su (mean $\pm$ SD) was detectable in plasma of patients 4 h after dose 1, and higher levels were observed after dose 9 ( $249.9 \pm 123.4$  ng/mL). This method may be used in clinical studies investigating the role of Su on adverse events and drug interactions associated with Bu therapy.

**Keywords** GC–MS/MS · Sulfolane · Busulfan · Metabolite · Pharmacokinetics · Therapeutic drug monitoring · Plasma

François Versace and Chakradhara Rao S Uppugunduri contributed equally to this work.

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F. Versace · P. Mangin · C. Staub  
Unit of Toxicology, University Center of Legal Medicine,  
1211 Geneva, Switzerland

C. R. S. Uppugunduri · F. Gumy-Pause · M. Ansari (✉)  
Department of Pediatrics, Onco-hematology Unit,  
University Hospital of Geneva,  
1211 Geneva, Switzerland  
e-mail: Marc.Ansari@hcuge.ch

M. Krajinovic · Y. Théorêt  
Department of Pharmacology, CHU Sainte Justine,  
University of Montréal,  
H3C 3J7 Montréal, Canada

## Introduction

Busulfan (Bu) is one of the most commonly used alkylating agents in the myeloablative conditioning regimen before hematopoietic stem cell transplantation [1, 2]. Hepatic veno-occlusive disease, pulmonary interstitial fibrosis, mucositis, and convulsions are the common adverse events seen with Bu therapy. Studies have shown association of these adverse events with high plasma levels of Bu, especially in children [3, 4]. However, occurrence of these events cannot be completely explained by the Bu plasma levels [5]. Indeed, the mechanisms of the adverse events are not clearly understood, and the role of Bu and its reactive metabolites on elucidating these events or their interaction with co-administered drugs is not clearly known.

Bu is metabolized by liver cytosolic glutathione-*S*-transferase enzymes (GST isoforms GSTA1-1, GSTM1-1, and GSTP1-1) to form a positively charged conjugate known as a sulfonium ion conjugate [ $\gamma$ -glutamyl- $\beta$ -(*S*-tetrahydrothiophenium)-alanyl-glycine], which dissociates non-enzymatically at alkaline pH and 37 °C or by enzymatic reaction catalyzed by cystathionine- $\gamma$ -lyase to tetrahydrothiophene (THT), pyruvate, and ammonia [6, 7]. THT is a lipophilic, volatile heterocyclic small molecule that gets oxidized to THT1,1-dioxide or sulfolane (Su) via the formation of THT,1-oxide [8, 9]. THT, 1-oxide, Su, and its oxidation product 3-hydroxy Su have been identified as the urinary metabolites of Bu in humans [10]. It is presumed that several cytochrome P 450 enzymes (CYP isoforms) might play a role in the oxidation of THT to Su and in the formation of 3-hydroxy Su. It is also presumed that these metabolites might play a role in altering the metabolism of cyclophosphamide administered after Bu. However, there are no data available on CYP isoforms catalyzing these oxidation reactions or on the distribution and fate of the metabolites, due to the lack of established analytical methods.

Two analytical methods for THT measurement have been described in the literature, and applied to *in vitro* and *in vivo* experiments [11, 12]. Nevertheless, the use of these methods for plasma level measurements of THT is tedious, and the detection of THT in samples collected during clinical studies is problematic due to its volatile nature. THT 1,1-dioxide or Su is a small molecule that is relatively stable compared to THT and is an immediate metabolite formed after oxidation of THT1-oxide. Su undergoes oxidation to 3-hydroxy Su, which is excreted in urine due to its hydrophilic nature [10]. Su is thus an important relatively stable intermediate metabolite, and its levels in plasma may be indicative of the metabolic fate of Bu. Even though a method for Su determination from ground water was described for environmental investigations [13], to the best of our knowledge, no method has been described in the literature for Su quantification in the plasma of patients receiving intravenous Bu.

In this article, we describe a novel gas chromatography–tandem mass spectrometric (GC–MS/MS) method for the measurement of Su in human plasma micro samples. This method can be easily implemented along with Bu measurements during routine monitoring without requiring additional sample volume using the same analytical platform. We believe that this strategy represents an efficient tool for the evaluation of metabolite distribution in patients receiving higher doses of Bu.

## Material and methods

### Samples collection

Clinical samples were obtained from patients who underwent hematopoietic stem cell transplantation in the Department of

Pediatric Hematology and Oncology of the University Children's Hospital, Geneva, Switzerland ( $n=3$ ) and in the Department of Pediatrics of CHU Sainte Justine, Montreal, Canada ( $n=56$ ). The protocol was approved by the local hospital ethics committee, and each patient/parent signed a consent form.

These samples were collected for routine therapeutic drug monitoring of Bu, as explained elsewhere [14], under an ongoing European Group for Bone Marrow Transplant Bu polymorphism study. The study was conducted according to the revised Declarations of Helsinki, the standards of Good Clinical Practice, and the Swiss regulatory requirements. The blank plasma used for calibration and validation was composed of Bu and Su-free pooled plasma provided by the University Hospital of Geneva.

### Chemicals and reagents

Reference Su (>99.5 % pure), Bu, and 2,3,5,6-Tetrafluorothiophenol (TFTP) were purchased from Sigma-Aldrich (Steinheim, Germany). Su-d8 (99 % atoms deuterated Su) was obtained from ARMAR Chemicals (Döttingen, Switzerland), and Bu-d8 was provided by Toronto Research Chemicals Inc. (Toronto, Canada). These reference materials were stored at room temperature. Ethyl acetate and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Isopropanol (>99.5 % pure) solvent, dried over molecular sieves, was obtained from Fluka (Buchs, Switzerland).

Stock solutions of Su and Su-d8 were prepared by weighing 10 mg (corresponding to 8  $\mu$ L) of pure substances into 10 mL of Milli-Q water. As both Su and Su-d8 are solids at room temperature (melting point, 27.5 °C), small fragments of reference materials were thawed by heating at 35 °C. Working standard solutions [0.1 and 1  $\mu$ g/mL for Su, 10  $\mu$ g/mL for Su-d8 as the internal standard (IS1)] were prepared by dilution in Milli-Q water. Stock and working standard solutions were freshly prepared when needed. A stock solution of Bu was prepared by weighing 10 mg of pure substance in 10 mL of acetonitrile. A stock solution of Bu-d8 was prepared by adding 1 mL of acetonitrile into the original flask containing 2.5 mg of pure substance. Working standard solutions [1 and 10  $\mu$ g/mL for Bu, 2.5  $\mu$ g/mL for Bu-d8 (IS2)] were then prepared in Milli-Q water. Stock and working standard solutions were stored at –20 °C.

### Preparation of calibrators

Working standard solutions were used to spike drug-free plasma to reach the concentrations of interest. Calibration samples were prepared by adding the corresponding volume of adequate standard solution in 100  $\mu$ L of blank plasma. Final concentrations of 500 ng/mL for Su-d8 (IS1) and 250 ng/mL for Bu-d8 (IS2) were achieved by adding 5 and 10  $\mu$ L of the corresponding working standard solutions, respectively.

### Sample pretreatment

After spiking with ISs and adding 50  $\mu\text{L}$  of 1 M NaOH, 100  $\mu\text{L}$  of plasma was extracted with 500  $\mu\text{L}$  of ethyl acetate by horizontal shaking (20 moves/s) for 5 min. After centrifugation (14,800 rpm, 1 min), the organic layer was removed. A 100- $\mu\text{L}$  aliquot was separated for Bu quantitation. A 50- $\mu\text{L}$  aliquot of isopropanol was added to the remaining 400  $\mu\text{L}$ , and the ethyl acetate was evaporated under a gentle flow of nitrogen at room temperature for approximately 10 min. A 1- $\mu\text{L}$  aliquot of the remaining 50- $\mu\text{L}$  isopropanolic residue was injected into the GC–MS/MS system.

The 100- $\mu\text{L}$  remaining aliquot for Bu quantitation was treated as described by Quernin et al., with the following alterations [15]. Briefly, 100  $\mu\text{L}$  of Milli-Q water, 25  $\mu\text{L}$  of 1 M NaOH, and 10  $\mu\text{L}$  of pure TFTP were added to the 100- $\mu\text{L}$  organic phase. After mixing, the derivatization was achieved by heating at 60  $^{\circ}\text{C}$  for 30 min. After cooling, 200  $\mu\text{L}$  of ethyl acetate was added to the mixture for back extraction by strong horizontal shaking (20 moves/s). After centrifugation, the organic layer was evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was reconstituted in 50  $\mu\text{L}$  of ethyl acetate, and 1  $\mu\text{L}$  was injected into the GC–MS/MS system. The overall extraction procedure is summarized in the Electronic supplementary material (ESM) Fig. 1.

### GC–MS/MS analysis

Analyses were performed on a Varian CP 3800 gas chromatograph (Walnut Creek, USA), equipped with a Varian CP-8400 autosampler and a Varian 300-MS triple quadrupole mass spectrometer. Data acquisition and analysis were performed through the Varian MS workstation software (version 6.9.3). Chromatographic separation was made on a Zebron fused-silica capillary column (ZB-5 MS, 15 m $\times$ 0.25 mm i.d., film thickness 0.25  $\mu\text{m}$ , with a 10-m inert guard column) from Phenomenex (Torrance, USA). High-purity helium 50 (99.999 %) was used as the carrier gas with a constant flow of 1 mL/min. The injector temperature was set to 250  $^{\circ}\text{C}$ , and the injection was made in splitless mode, with the injection purge valve remaining closed for 1 min.

For Su quantitation, the initial oven temperature was set to 60  $^{\circ}\text{C}$  and held for 0.1 min. It was then increased to 82  $^{\circ}\text{C}$  at 70  $^{\circ}\text{C}/\text{min}$  and held for 1 min, increased to 105  $^{\circ}\text{C}$  at 70  $^{\circ}\text{C}/\text{min}$  and held for 0.5 min, increased to 280  $^{\circ}\text{C}$  at 70  $^{\circ}\text{C}/\text{min}$ , and finally maintained for 0.26 min. For Bu quantitation, the initial oven temperature was set to 60  $^{\circ}\text{C}$  and held for 0.5 min. It was increased to 200  $^{\circ}\text{C}$  at 70  $^{\circ}\text{C}/\text{min}$  and then to 280  $^{\circ}\text{C}$  at 50  $^{\circ}\text{C}/\text{min}$ . The final temperature was maintained for 0.90 min. Both GC runs lasted 5 min only. The transfer line, manifold, and ion source were heated at 275, 40, and

200  $^{\circ}\text{C}$ , respectively. Ionization was achieved in electronic impact mode (EI) at 70 eV. The MS/MS experiments were based on collisionally induced dissociation, which occurred in the collision cell with an argon pressure of 1.5 mTorr. Two selected reaction monitoring transitions were considered for each analyte (Table 1). They were chosen according to their selectivity and sensitivity.

### Validation procedure

The method was validated according to the European Medicines Agency guidelines [16]. The validation was carried out over three nonconsecutive days ( $p=3$ ). A statistical treatment of the results, based on variance analysis, allowed a determination of the precision and trueness of the method for the selected concentration levels.

A validation day consisted of independently preparing calibrators and quality control samples (QC) with both analytes spiked in the same sample. Calibrators were prepared in duplicate ( $n=2$ ) at five concentrations levels, i.e., 20, 50, 100, 200, and 500 ng/mL for Su and 50, 200, 500, 1,000, and 2,000 ng/mL for Bu. These samples allowed the determination of the response functions of both analytes, calculated with the analyte to internal standard ratios. The calibration range was chosen according to the concentrations observed in a patient's samples during the pre-validation step. The QC samples were prepared independently in quadruplicate ( $n=4$ ) at four concentration levels, i.e., 20, 40, 100, and 400 ng/mL for Su and 100, 200, 500, and 2,000 ng/mL for Bu.

The spiked samples were then treated as described earlier. The validation procedure permitted the determination of trueness, precision, linearity, and the limits of quantification. The lower limit of quantification (LLOQ) was determined as the lowest QC concentration with trueness and precision under  $\pm 20\%$ . The overall extraction recovery of Su was evaluated by comparison of the extracted samples with a non-extracted isopropanolic solution.

**Table 1** MS/MS method parameters for busulfan and sulfolane

Compound	Molecular mass (g/mol)	Retention time (min)	Q1 $\rightarrow$ Q3 ( $m/z$ )	Collision energy (eV)
di-TFTP butane d8	426	3.91	<u>426<math>\rightarrow</math>245</u>	10
di-TFTP butane	418	3.92	418 $\rightarrow$ 237	10
			<u>237<math>\rightarrow</math>195</u>	10
Sulfolane d8	128	2.88	<u>128<math>\rightarrow</math>62</u>	10
Sulfolane	120	2.90	<u>120<math>\rightarrow</math>55</u>	10
			120 $\rightarrow$ 41	15

Quantitative transitions are underlined; the others were used for identification

d8 deuterated; TFTP 2,3,5,6-tetrafluorothiophenol

## Recovery and stability tests

The stability of Bu in frozen plasma has already been investigated, and no further experiments were performed in this study [17]. The extraction procedure was also taken from the literature and was not re-evaluated [15]. The short-term stability of Su in plasma was evaluated at 100 and 400 ng/mL levels. These samples were analyzed in triplicate on the day 0, and aliquots were stored at  $-20^{\circ}\text{C}$ . They were then analyzed after 7, 14, 21 ( $n=1$ ), and 90 days ( $n=3$ ). The extraction recovery of Su was evaluated by comparing the Su peak areas of the extracted spiked plasma ( $n=6$ ) with a corresponding isopropanolic solution injected six times.

## Results and discussion

Two difficulties had to be overcome in the development of a quantitative analytical method for Su along with Bu in pediatric plasma samples. First, Bu is not volatile enough for gas chromatography. Several derivatization protocols have been proposed to avoid this incompatibility [15, 18–20], and liquid chromatography methods coupled to mass spectrometry are common [21, 22]. However, the objective of this study was to quantify the Su in the patients' single plasma sample along with Bu, which seemed to be more feasible by GC due to the nature of Su. The Bu extraction and derivatization procedure described by Querin et al. was chosen and adapted for this purpose [15].

The physical properties of Su were also problematic for sample preparation. Evaporation to dryness of the working standard solutions or extracts followed in conventional procedures could not be performed. Indeed, incoherent peak areas or even complete disappearance of Su was observed during the pre-validation step after evaporation of the methanolic standard solutions and extracts to dryness using nitrogen or evaporating under vacuum. This was probably due to the partial or complete evaporation or sublimation of Su

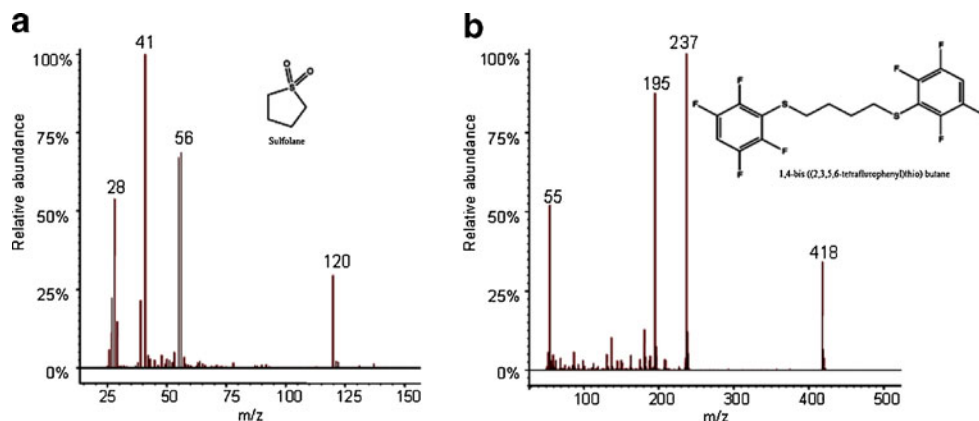
occurring after solvent elimination at room temperature. This problem was solved by spiking the plasma sample directly with aqueous solutions of Su and Su-d8, and adding isopropanol in the ethyl acetate extract before evaporation. Isopropanol is fully miscible in ethyl acetate in the used proportions and has a lower volatility. This property allows for better control of the evaporation of the extracts. Moreover, isopropanol was found to be a suitable solvent for GC. The extraction recovery was estimated to be 93 % ( $n=6$ , 6 % RSD).

The second difficulty was the small amount sample material because Bu derivatization step seemed to be incompatible with Su analysis. Indeed, no Su peak could be detected after Bu derivatization of the spiked plasma. Thus, two separated sample preparations had to be investigated, preferably from a single plasma sample of reasonable volume. Nevertheless, concentrations found in real clinical samples during the pre-validation step were sufficiently high to suit the instrument sensitivity without extensive pre-concentration. Therefore, a volume of 100  $\mu\text{L}$  of plasma, split into two fractions after the addition of organic solvent, was found to be a good compromise between the sensitivity needs and the scarcity of available sample.

## GC–MS/MS analysis of busulfan and sulfolane

Two MS/MS transitions were investigated for Su, although its nominal mass was small (120 u). As illustrated in Fig. 1a, the EI of Su showed predominant fragment ions of mass to charge ratio ( $m/z$ ) of 41, 56, and 28, corresponding to the loss of  $\text{SO}_2$  and fragmentation of the  $(\text{CH}_2)_4$  chain. The molecular ion ( $m/z$  120) was abundant enough (28 %) to be selected as the precursor ion for two MS/MS transitions, which avoided the selection of very low mass fragments. This produced better selectivity and a substantial increase in the sensitivity of the method. In the case of Bu converted to di-TFTP butane, the molecular ion ( $m/z$  418) was also abundant enough (34 %) to develop one MS/MS identification transition (Fig. 1b). The most abundant fragment ( $m/z$

**Fig. 1** Mass spectra of sulfolane (a) and busulfan derivatized to 1,4-TFTP-butane (b) after electron impact ionization. X-axis represents mass to charge ratio ( $m/z$ ), and y-axis represents relative abundance in percentage and is related to the number of times an ion of a specific  $m/z$  ratio strikes the detector



**Table 2** Validation summary of quantification method for sulfolane

Analyte	Concentration of quality control sample (ng/mL)	Trueness (%) ( $k=4$ ; $n=4$ ; $p=3$ )	Repeatability (R.S.D. %) ( $k=4$ ; $n=4$ ; $p=3$ )	Intermediate precision (R.S.D. %) ( $k=4$ ; $n=4$ ; $p=3$ )
Sulfolane	20	118.0	4.4	11.4
	40	100.3	6.8	9.2
	100	102.1	7.1	11.7
	400	109.3	7.7	11.6
Busulfan	100	113.2	4.9	7.2
	200	109.2	5.4	6.7
	500	97.6	6.9	9.4
	2,000	106.1	10.5	10.5
Regression equations for sulfolane	Day 1	$Y=0.0017X-0.0262$		$R^2=0.985$
	Day 2	$Y=0.0018X-0.0180$		$R^2=0.989$
	Day 3	$Y=0.0017X-0.0265$		$R^2=0.972$
Regression equations for busulfan	Day 1	$Y=0.0009X-0.0647$		$R^2=0.994$
	Day 2	$Y=0.0007X+0.0434$		$R^2=0.997$
	Day 3	$Y=0.0007X+0.0272$		$R^2=0.980$

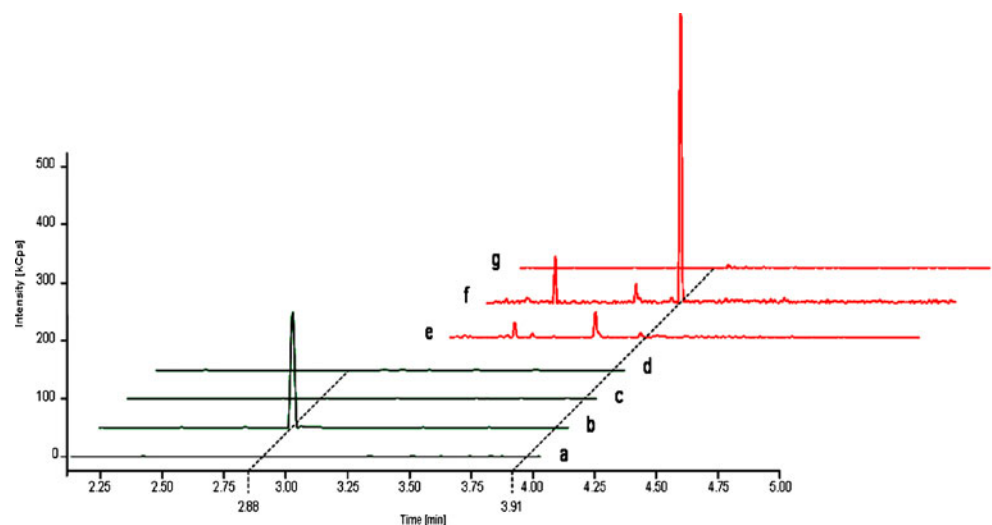
$k$  is the number of concentration levels,  $n$  is the number of repetitions by level, and  $p$  is the number of nonconsecutive days. The regression equations used for calculations of Su after  $1/X^2$  weighting for each of the 3 days generated with calibrator samples run in duplicates at five concentration levels (20, 50, 100, 200, and 500 ng/mL). Regression equations for Bu were  $1/X$  weighted generated with calibrator samples run in duplicates at five concentration levels (50, 200, 500, 1,000, and 2,000 ng/mL). The measured concentration values at each quality control concentration level include mean $\pm$ SD of values injected in quadruplicates on each of the three validation days. This validation was performed using the same plasma sample in which both Bu and Su were spiked for quantification by separate chromatography runs. Repeatability represents the intra-day variability, whereas intermediate precision represents inter-day variability

237), corresponding to the loss of one TFTP group, was selected as the precursor ion for a quantitative transition.

### Validation

During the pre-validation step, it was determined that a  $1/X^2$  and  $1/X$  weighted linear regression models were the most suitable for Su and Bu, respectively, giving the most representative response function. Validation data are summarized in Table 2.

**Fig. 2** Typical current ion chromatograms with the sulfolane GC–MS/MS method (green) and busulfan GC–MS/MS method (red), showing injections of extracted blank plasma (a, e), extracted spiked blank plasma (b, f), and isopropanol (c, g). d An injection of a 10- $\mu$ g/mL derivatized busulfan solution



### Selectivity and carryover

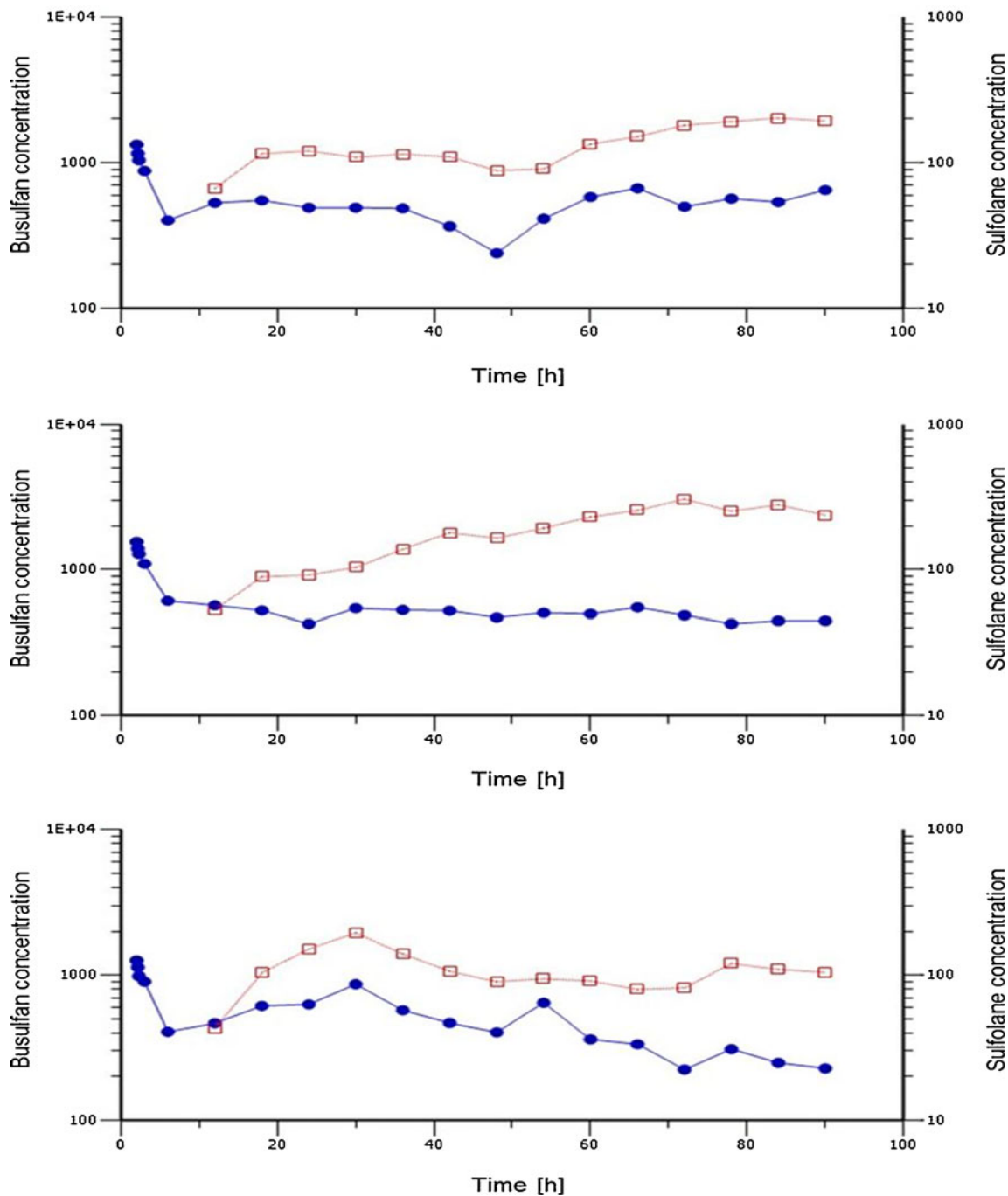
Selectivity was evaluated by injecting five different blank plasma samples, including the pooled plasma and patients' plasma prior to infusion of Bu, to check the absence of an interfering peak at the retention times of interest. Figure 2a, e shows the typical chromatograms of extracted blank plasma, demonstrating the absence of interferences for Bu and Su transitions. Figure 2b, f shows chromatograms for blank plasma spiked with Bu (200 ng/mL) and Su (20 ng/mL). No



interfering peak was observed in the transitions of Su when injecting highly concentrated (10  $\mu\text{g}/\text{mL}$ ) derivatized Bu, as shown in Fig. 2d. Carryover was also investigated by injected isopropanol after four injections of the highest QC. The chromatograms showed no peaks for both analytes (Fig. 2c, g).

Linearity, trueness, precision, and lower limit of quantification

Linearity was calculated by fitting the back-calculated concentrations of the QCs as a function of the introduced concentrations and by applying the linear regression model



**Fig. 3** Time vs. plasma concentration profiles of sulfolane and busulfan measured in three different individuals. The sulfolane (red squares) and busulfan (blue circles) levels were measured at 0, 15, 30 min, 1, 4 h after the first dose and 4 h after each dose infusion up to the 15th dose. Sulfolane levels were detected at 4 h after the first dose infusion,

but were below the quantifiable levels, hence not included in the graph. X-axis represents time in hours, and y1-axis represents busulfan concentration in nanograms per milliliter, y2-axis represents sulfolane concentration in nanograms per milliliter. Both y1 (left) and y2 (right) axes were in logarithmic scale

based on the least-squares method. The regression equations for used both analytes and the coefficient of determinations ( $R^2$ ) are given in Table 2. The trueness and precision were determined for each analyte by injecting independent QC samples at different concentration levels and by calculation of the difference between measured and theoretical concentrations.

For Su, the trueness at 20 ng/mL (lowest QC) was found to be 118.0 % ( $<\pm 20$  %) and in the range of 100.3–109.3 % for the other QCs ( $<\pm 15$  %; Table 2). For Bu, the trueness was found to be 113.2 % ( $<\pm 15$  %) at the lowest QC level and in the range of 97.6–109.2 % for the other levels ( $<\pm 10$  %). Precision values were assessed by calculating the relative standard deviations (RSD). The intra-day variability was expressed as repeatability and inter-day variability was expressed as the intermediate precision. These values were less than  $\pm 15$  % (acceptable levels) for all the concentrations tested (Table 2). The lowest QC concentration level for Su (20 ng/mL) and Bu (100 ng/mL) has trueness and precision less than  $\pm 20$  %, and hence defined as the LLOQ. These LLOQ values were fully adequate to quantify both analytes in patients' plasma receiving high doses of Bu. All the calibrator samples had trueness and precision profiles  $<\pm 15$  %. Validation parameters for Bu quantification were in accordance with previously described literature [15, 19–22]. To our knowledge, no method for quantitation of Su in patients' plasma had been published before, and no comparison could be made.

#### Stability of sulfolane in plasma samples and extracts

We explored short-term stability at room temperature and  $-20$  °C. Extracts were found to be stable for more than 12 h at room temperature (data not shown). This is much longer than typical analysis sequence duration. Moreover, no significant decrease in Su concentration was observed after 90 days at  $-20$  °C (See ESM Fig. 2).

#### System stability

The analysis of a large number of samples allowed us to check the stability of the analytical system (ESM Fig. 3). The variation of area of the IS1 over 79 successive injections is relatively high (25 % RSD), partly due to the variation in the volume of organic layer removed from the extraction tube and to the variation of the final volume after evaporation of the ethyl acetate fraction, leading to disparity in concentration factors over the samples. However, no significant difference ( $t$  test) was observed between the means of the first and the last ten injections, indicating good robustness of the analytical method. Hence, this method showed to be suitable for large-scale clinical studies.

**Table 3** Sulfolane and busulfan levels measured in real patient samples

Sample	Sulfolane (ng/mL)	Busulfan (ng/mL)
Before dose 7 infusion ( $n=56$ )	181.9 $\pm$ 80.9 (179.1)	393.4 $\pm$ 184.8 (365.7)
End of dose 9 infusion ( $n=54$ )	249.9 $\pm$ 123.4 (239.9)	1262.5 $\pm$ 538.5 (1196.3)

All values are mentioned as mean $\pm$ SD (median). The plasma levels of Su measured at 4 h after the first dose may not represent exact quantifiable levels since the lower limit of quantification is 20 ng/mL, and the values were around 20 ng/mL and are not mentioned ( $n=46$ ) here due to the lack of analytical and clinical significance, but Su can be detected 4 h after starting busulfan first dose infusion

#### Application to clinical studies

Figure 3 shows the concentration–time profiles of Su and Bu in three children receiving intravenous Bu (0.8 mg/kg) over a 2-h period in a conventional 16-dose protocol [14]. The Bu concentrations presented a typical pattern, with a rapid increase and decrease of plasmatic concentrations corresponding to the first dose intravenous infusion. Bu levels were relatively stable in individuals measured at 4 h (trough level) after each dose infusion up to the 15th dose. The Su concentrations showed an interesting profile. A constant increase in plasma concentrations over the doses was observed. Sulfolane was not detectable until the end of the 4 h following the first dose infusion, but it was readily quantifiable after the second dose infusion. Using large volumes of injections might have improved the sensitivity of the method and allowed for an earlier detection of Su. However, this was found to be unnecessary for this study as the concentrations rapidly reached quantifiable levels in all three patients. Based on these preliminary observations, three time points were chosen (4 h after dose 1, 4 h after dose 6, and 0 min after dose 9 infusion) to measure the Su levels in a large number of patients. The levels of Su and Bu from these samples at 4 h after dose 6 and 0 min after dose 9 infusions are presented in Table 3. Su was detectable at 4 h after dose 1, but its levels were around the LLOQ (20 ng/mL), and may not have any analytical or clinical significance, hence were not discussed here. Higher levels of Su were observed after dose 9 infusion. All the plasma samples used for analysis were stored for more than 6 months (up to 3 years) at  $-80$  °C. We also observed interindividual variability in the levels of both Bu and Su. Thus, by using this method, one can investigate the role of Su in determining adverse events and drug interactions during Bu treatment using plasma samples collected for routine Bu monitoring and during clinical studies.

#### Conclusion

This novel method presented in this article allowed the determination of Su in plasma micro samples. Validation

data showed a satisfactory degree of sensitivity, trueness, and precision. The extraction procedure for Su is fast and simple, and can easily be included in the routine analysis of Bu as no extra volume of plasma is required. Clinical studies may adopt this method for investigating the Bu metabolic fate and its mechanisms of toxicity. The method described here may be used for understanding the kinetics of Su in subjects receiving Bu infusion and its role in the adverse effects and drug interactions seen with Bu therapy.

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**Conflicts of interest** The authors declare no competing financial interests.

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