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Quantification of busulfan in plasma by liquid chromatography–ion spray mass spectrometry
Application to pharmacokinetic studies in children

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Abstract

Optimisation of busulfan dosage in patients undergoing bone marrow transplantation is recommended in order to reduce toxic effects associated with high drug exposure. A new method was developed coupling liquid chromatography with mass spectrometry (LC–MS) and was validated for the determination of busulfan concentrations in plasma. Recovery was 86.7%, the limit of detection was 2.5 ng/ml and linearity ranged from 5 to 2500 ng/ml. The correlation between the busulfan concentrations measured by our previously published HPLC–UV method and the new HPLC–MS method was highly significant ($P<0.0001$). Sample volume was reduced and the method was rapid, sensitive and less expensive than the methods previously used in our laboratory. This method was used to determine the pharmacokinetic parameters of busulfan after the first administration of 1 mg/kg orally, in 13 children receiving the drug as part of the preparative regimen for bone marrow transplantation. Our results were similar to previously reported data. They showed that the apparent oral clearance of busulfan was 0.299 ± 0.08 l/h/kg, and that it was significantly higher ($P=0.02$) in patients below the age of 5 years than in older children. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Busulfan (1,4-butanediol dimethanesulfonate) is a bifunctional alkylating agent commonly administered in preparative chemotherapy regimens for bone marrow transplantation for patients with haematological malignancies and non-malignant disorders. It is usually given at the oral dose of 1 mg/kg every 6 h for 16 doses in association with cyclophosphamide. The pharmacokinetics of the drug administered orally were highly variable in paediatric patients, particularly in young children and were primarily related to variable absorption and clearance [1–3]. The toxic effects of busulfan include hepatic veno-occlusive diseases and mucositis, which were strongly related to high drug exposure measured by the steady-state plasma concentrations

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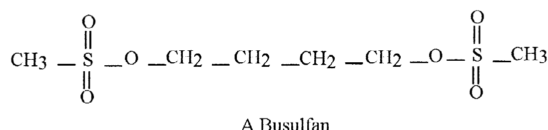
[4] and/or area under the curve of busulfan [5]. Additional risk factors for severe veno-occlusive disease include multiple medications given during the peritransplant period, high pretransplant transaminases and alkaline phosphatases and the timing of cyclophosphamide administration [5]. In addition, high busulfan concentrations have been independently associated with overall survival [4] while the association between low concentrations and increased risk of rejection remains controversial [6]. However, because decreased toxicity could be associated with decreased efficacy, individual dosage adjustment is now advocated [1,2,4,7,8] but requires the rapid determination of busulfan plasma concentrations [9] after the first dose [2,3] and/or predose pharmacokinetics to predict steady-state plasma concentrations [10]. In order to achieve this, new methods to quantify busulfan in small volumes of plasma should be developed. We validated a new high-performance liquid chromatography with mass spectrometry detection (HLPC–MS) method that we compared with methods already used in our laboratory [11,12]. Using this method, we performed a pharmacokinetic study of busulfan in 13 children.

2. Experimental

2.1. Chemicals

Busulfan (Fig. 1A) was obtained from Aldrich (Saint Quentin Fallavier, France). Busulfan-d_8 (Fig. 1B) was synthesised by Eurisotop (Saint Aubin, France). All solvents were of analytical grade.



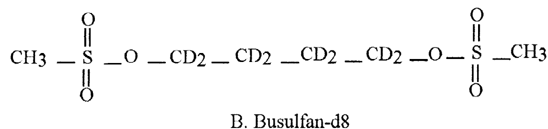


Fig. 1. Chemical structure of busulfan (A) and busulfan-d_8 (B).

2.2. HPLC–MS system

The HPLC–MS system consisted of a series 200, pump and refrigerated autosampler with a 20-μl loop (Perkin Elmer, Norwalk, CT, USA), a PE Sciex Api 150EX quadripole mass spectrometer (PE Sciex, Toronto, Ontario, Canada) with a TurboIonSpray interface. The pump equipped with a vacuum membrane degasser delivered the mobile phase at the flow-rate of 200 μl/min into a phenyl 5-μm particle size (250×2 mm) Stability column (CIL Cluzeau, Sainte Foy La Grande, France). The mobile phase consisted of methanol–10 mM ammonium acetate (60:40, v/v) and was split with a ratio of 1/5 at the entrance of the mass spectrometer. Data were acquired in positive ion mode with an ion spray probe voltage of 5500 V. The parameter settings for nebulizer and curtain gasses were 8 and 10 units, respectively. The MS conditions for declustering potential, ring voltage and entrance quadripole potential were 11, 110 and −3 V, respectively.

2.3. Busulfan assay in plasma

Two stock solutions of busulfan (500 and 10 μg/ml) and a stock solution of busulfan-d_8 (50 μg/ml) were prepared in acetone and stored in 500-μl aliquots at −20°C. Calibration standards (5–2500 ng/ml) were prepared daily by drying in nitrogen at 40°C appropriate dilutions of the stock solution (500 μg/ml) and diluting the dry residue in 200 μl of drug-free plasma. For quality controls (400 and 700 ng/ml), 80 and 140 μl of a dilution of stock solution of busulfan (10 μg/ml) were dried after 200 μl of drug-free plasma were added. Quality controls were stored at −20°C.

Plasma sample (200 μl) diluted in 300 μl of deionised water and spiked with 100 μl of internal standard (busulfan-d_8 250 ng/ml in acetone) were extracted with 2 ml of ethyl acetate. The organic phase was dried in nitrogen and the residue was dissolved in 500 μl of mobile phase.

2.4. Comparison of the LC–UV and GC–MS methods using TFTP derivatisation, with LC–MS method

In the previous HPLC method for busulfan assay [12], plasma samples (1 ml) without I.S. were

extracted with toluene and busulfan was derivatised for 2 h by 2,3,5,6-tetrafluorothiophenol (TFTP). Derivatised busulfan (di-TFTP-butane) was reextracted with toluene and analysed by HPLC–UV in a chromatographic run time of 40 min.

The same procedure, except for the use of an I.S. (busulfan-d_8) and ethyl acetate for the two extractions, was used for the GC–MS method [11]. A summary of the three methods is given in Fig. 2.

2.5. Pharmacokinetic study

Thirteen paediatric patients, aged 1–12 years (median 7 years) were studied. They received busulfan (1 mg/kg every 6 h for 16 doses) as part of the conditioning regimen before bone marrow transplantation for acute leukaemia. The pharmacokinetic study was performed after the first administration of the drug. Eight blood samples were collected on ice in heparinised tubes in the 6 h following drug intake. Blood was immediately centrifuged and plasma was kept at −20°C until analysis.

The following pharmacokinetic parameters were calculated: area under the plasma concentration versus time curve (AUC_{0-6}) using the trapezoidal rule and extrapolated to infinity ($AUC_{0-\infty}$) using the elimination rate constant (K_{el}), apparent oral clearance (Cl/F) where F is the bioavailability, elimination half-life ($T_{1/2}$), concentration at steady-state (C_{ss}) calculated from the following equation $C_{ss} = AUC_{0-\infty}/\tau$ where τ is the interval between two administrations.

2.6. Statistical analysis

Results are expressed as mean and standard deviation. The comparison between the LC–UV and LC–MS methods was by the two-tailed Student's t-test. The statistical difference between pharmacokinetic

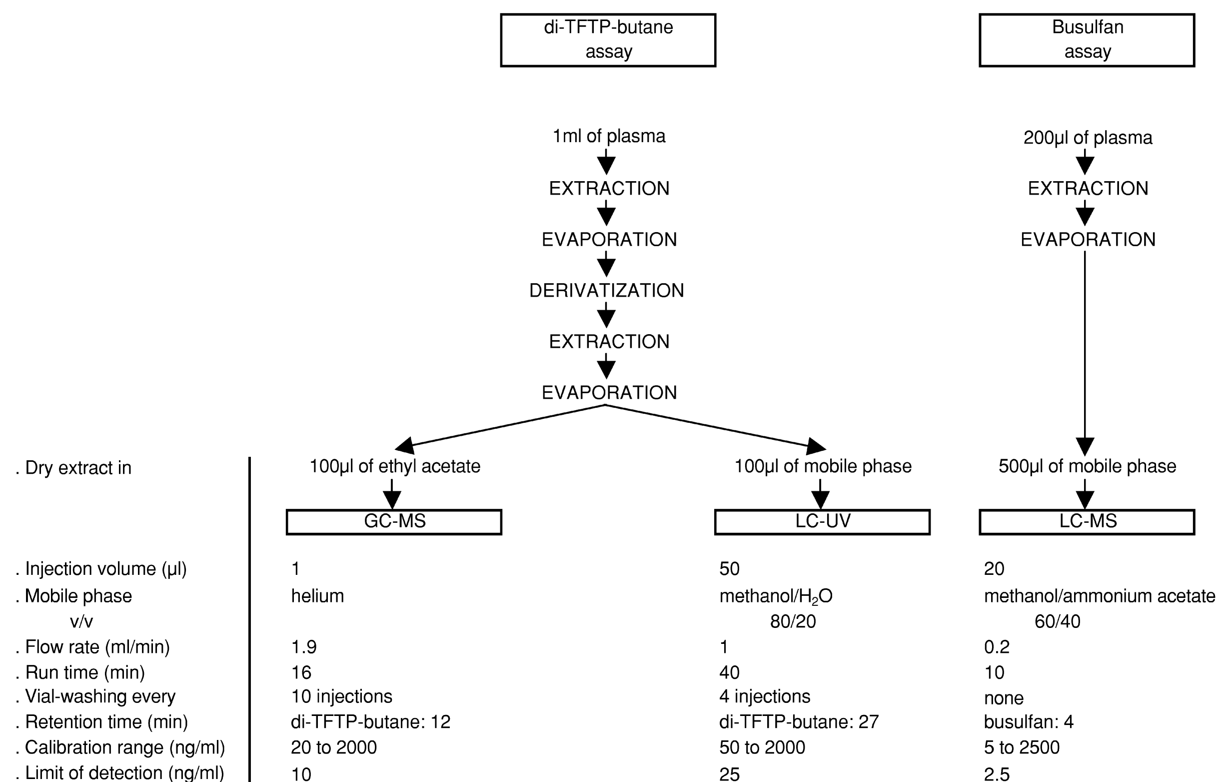


Fig. 2. Comparison of the GC–MS, LC–UV and LC–MS methods developed in our department.

parameters in the two groups was analysed by an unpaired Student's *t*-test.

3. Results

3.1. Mass spectra analysis

The full scan mass spectra of busulfan and busulfan-d_8 in ammonium acetate–methanol are presented in Fig. 3. Protonated molecules (MH^+) were not detected but ammonium-adducted molecules (MNH_4^+) at m/z 264 and m/z 272 for busulfan and busulfan-d_8, respectively, were predominant. The mass spectrometric parameters were optimised to obtain the higher signal for the MNH_4^+ ions and the analyses were performed using selected ion monitoring (SIM) at m/z 264 and 272 for busulfan

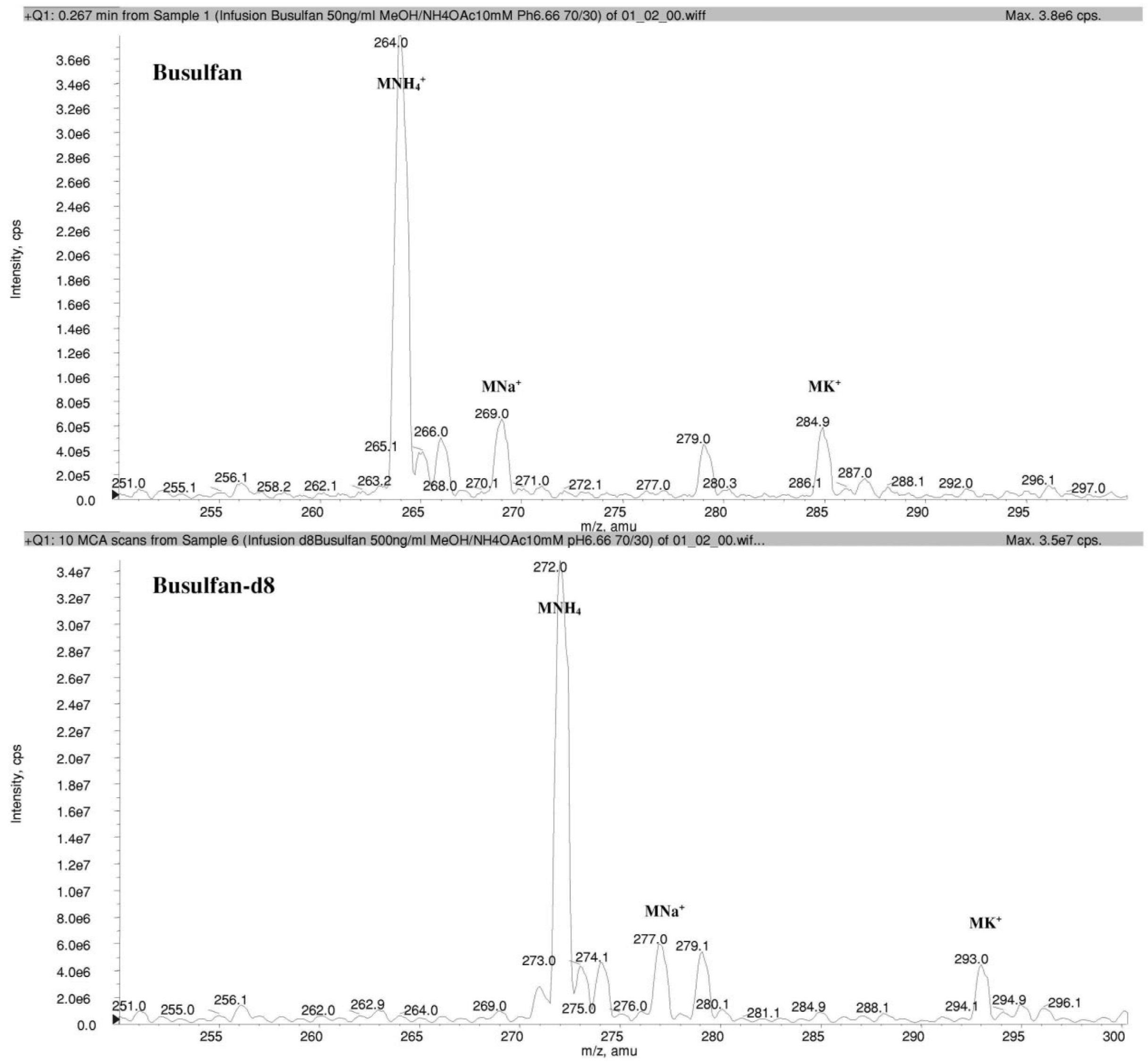


Fig. 3. Mass spectra of busulfan and busulfan-d_8 in ion spray mass spectrometry.

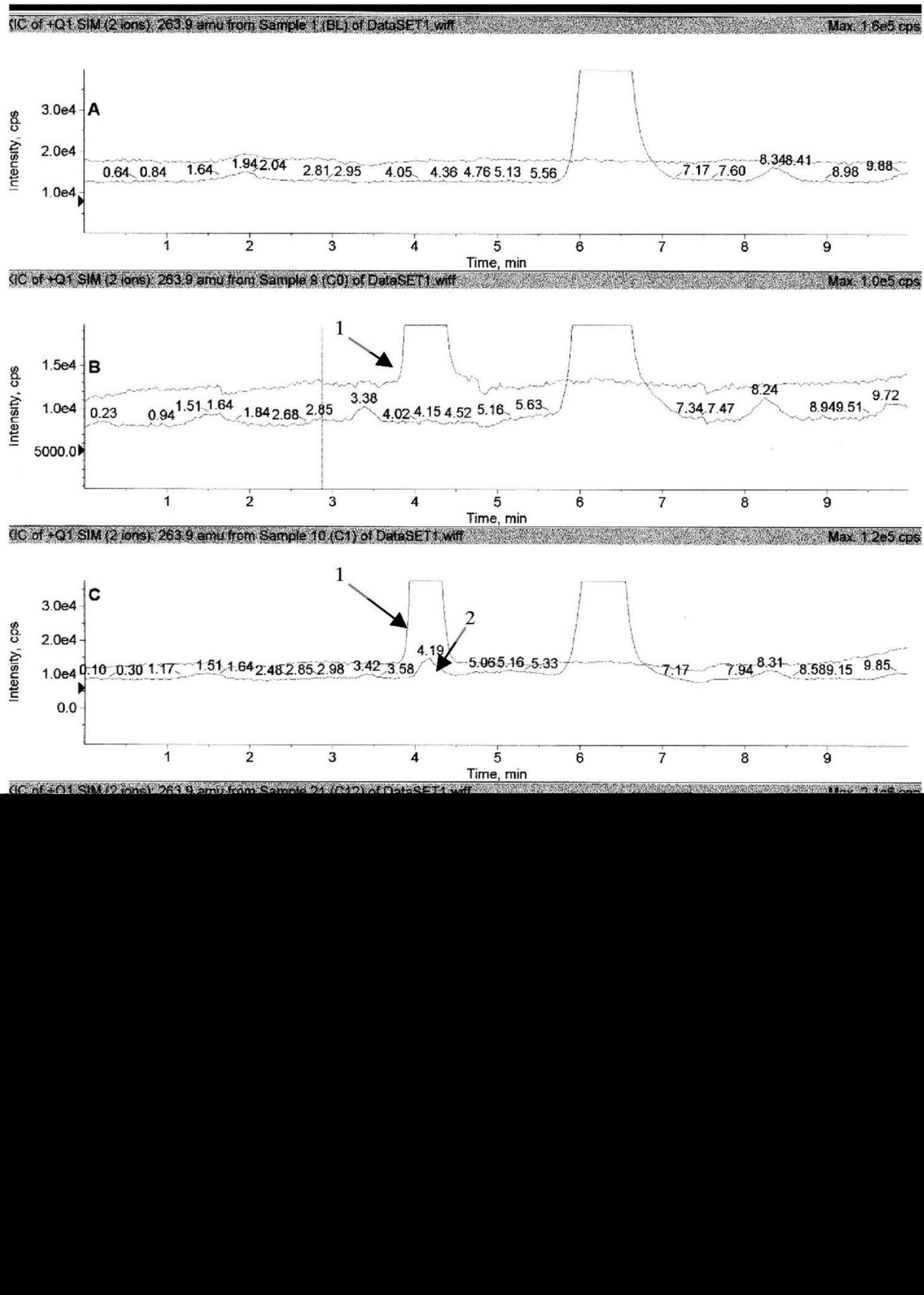


Fig. 4. Representative chromatograms obtained from a pool plasma (A) double blank, (B) single blank containing the internal standard, (C) lowest standard at the concentration of 5 ng/ml, (D) highest standard at the concentration of 2500 ng/ml. Representative chromatogram obtained from one patient (E) 30 min after the first oral administration of 1 mg/kg of busulfan, showing peak 1 corresponding to busulfan-d_8 and peak 2 corresponding to busulfan.

and busulfan-d_8, respectively. Mass spectra also showed ions at m/z (MNH_4^+ +2) corresponding to isotopic sulphur atoms (S_{34}) of busulfan and busulfan-d_8.

3.2. Assay performance

Under the chromatographic conditions described, the retention times for busulfan and busulfan-d_8 were 4.15 and 4.12 min, respectively, without an interference peak (Fig. 4).

The recoveries were calculated by comparing the peak areas ($n=4$) of busulfan (at 5, 50, 500, 1500 and 2500 ng/ml) and busulfan-d_8 (250 ng/ml) in plasma after extraction to equivalent amounts of these compounds injected directly into the LC–MS system. The extraction recoveries calculated were 86.7 and 91.2% for busulfan and busulfan-d_8, respectively. The calibration curve was linear over the concentration range of 5–2500 ng/ml. The limit of detection was 2.5 ng/ml for busulfan (signal-to-noise ratio of 3) by using a plasma sample size of 200 μl.

The intra-assay variability ($n=4$) was below 10% for all the concentrations tested and for the quality controls.

The inter-assay relative standard deviation ($n=7$) was below 10% for calibration standards and quality controls. The results are presented in Table 1.

3.3. Comparison with the LC–UV assay using derivatisation with tetrafluorothiophenol

Fifty-six plasma samples obtained from children treated with busulfan were analysed using the two methods. Busulfan concentrations ranged from 70 to 1100 ng/ml and 60 to 1061 ng/ml, respectively, with the LC–MS and the LC–UV methods. There was a significant correlation between busulfan concentrations measured by HPLC–MS and di-TFTP-butane concentrations ($r^2=0.89$, $P<0.0001$) (Fig. 5) and individual results were not different (Student's test for paired data, $P=0.064$). For each sample, the difference between the two readings (HPLC–UV and HPLC–MS) was calculated and the mean difference was 9.7%.

3.4. Pharmacokinetic results

The individual pharmacokinetic parameters of busulfan are presented Table 2. $AUC_{0-\infty}$ and C_{ss} were 3374±869 ng/ml h and 562±145 ng/ml,

Table 1
Quantification of busulfan in plasma: accuracy and precision of the LC–MS method

Busulfan conc. (ng/ml)	n		Mean concentration measured (ng/ml)		RSD (%)		Accuracy (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Calibration standards								
5	4	7	4.54	5	9.83	7.68	90.8	100
10	4	7	9.47	9.89	7.92	7.09	94.7	98.9
20	4	7	20.55	19.8	3.28	3.57	102.7	99
50	4	7	51.6	50.5	2.11	3.97	103.2	101
100	4	7	100.35	98.95	2.87	4.12	100.3	98.9
250	4	7	256.5	255.57	4.05	4.5	102.6	102.2
500	4	7	509.5	495.85	2.52	3.04	101.9	99.1
750	4	7	750.25	748.28	3.01	3.4	100	99.7
1000	4	7	1012.5	1020.5	2.18	2.98	101.2	102
1500	4	7	1512.5	1495.7	3.25	2.69	100.8	99.7
2000	4	7	1955	1972.8	2.26	3.61	97.7	98.6
2500	4	7	2500	2515.7	1.34	2.05	100	100.6
QC samples								
400	4	7	410.75	419.5	2.01	2.92	102.6	104.8
700	4	7	724.25	729.4	0.56	3.49	103.4	104.2

RSD, relative standard deviation; QC, quality controls.

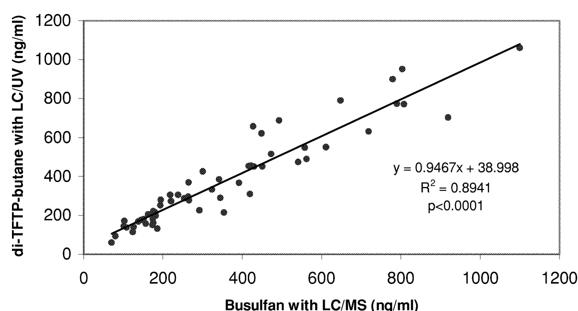


Fig. 5. Correlation between the concentrations of busulfan measured by LC–MS and di-TFTP-butane measured by LC–UV in 56 plasma samples.

respectively. Mean Cl/F was 0.299±0.08 l/h/kg and $T_{1/2}$ was 2.11±0.3 h.

The patients were divided into two groups according to age: group 1 (five patients below 5 years) and group 2 (eight patients over 5 years). Clearance was higher (0.358±0.09 l/h/kg versus 0.263±0.04 l/h/kg, $P=0.02$) in patients of group 1 compared to those of group 2. The other parameters were not significantly different between the two groups.

4. Discussion

The pharmacokinetics of busulfan are characterised by considerable inter-individual variability and dose-related toxicity. Busulfan is administered over 4 days and if busulfan concentrations can be determined following the first dose, dose adjustment may be possible during the following days of the preparative regimen for bone marrow transplantation.

Busulfan is a very polar compound with poor UV absorbency. Reference methods using GC–ECD [13–16] or GC–MS [11,17,18] or LC–UV [12,19–25] or LC–fluorescence detection [26] require derivatisation to quantify busulfan concentrations in plasma. We previously published two methods — GC–MS and LC–UV — using the same derivatization with TFTP [11,12]. The two methods were well correlated and were both sensitive, but they required at least 2 h for the derivatisation step. By forming a non polar compound with a specific UV absorbency, the derivatisation procedure with TFTP allowed the use of a GC technique and a UV absorbency detection in the GC–MS and LC–UV methods,

Table2
Pharmacokinetic parameters of busulfan after the first oral dose of 1 mg/kg in 13 paediatric patients

Patient no.	Age (years)	Dose (mg/kg)	AUC (ng/ml h)	Cl/F (l/h/kg)	$T_{1/2}$ (h)	C_{ss} (ng/ml)
1	1	1.14	3173	0.363	1.79	529
2	1	1.21	2487	0.483	2.13	414
3	1.5	1.4	4232	0.332	2.56	705
4	3.5	0.95	2579	0.367	1.63	430
5	4.5	1.16	4748	0.244	2.13	791
6	7	1.04	2983	0.348	2.11	497
7	7	0.83	2962	0.26	2.63	494
8[a]	8.5	0.4	1632	0.245	2.14	272
9	10.5	0.85	3807	0.221	2.34	635
10	10.5	0.93	3347	0.277	2.28	558
11	11.5	0.98	4142	0.237	2.15	690
12	11.5	0.98	4291	0.228	1.78	715
13	12	1	3477	0.288	1.8	580
Mean	6.9	0.99	3374	0.299	2.11	562
SD	4.2	0.24	869	0.08	0.3	145
RSD (%)	61	24	26	25	14	26
Group 1[b]	2.3±1.6	1.17±0.16	3443±1007	0.358±0.09	2.05±0.4	574±168
Group 2[b]	9.8±2.0	0.88±0.21	3330±843	0.263±0.04	2.15±0.3	555±140

$AUC_{0-\infty}$, area under the plasma concentration versus time curve extrapolated to infinity; F, bioavailability; Cl/F, apparent oral clearance; $T_{1/2}$, elimination half life; C_{ss}, concentration at steady state.

[a] Patient 8 had a Fanconi disease and received busulfan at the dose of 0.4 mg/kg.

[b] Values expressed as mean±SD.

respectively. We therefore supposed that if we coupled HPLC and MS, busulfan derivatisation would not be required.

Indeed, the new HPLC–ESI-MS method reported here has major advantages compared to previous methods. We eliminated derivatisation and extraction of the derivatised busulfan, shortening the assay procedure by 2.5 h. In addition, the run time is 10 min in the HPLC–MS method while it was 40 and 16 min in the HPLC–UV and GC–MS methods, respectively. Sample volume was reduced and the method was rapid, sensitive and less expensive.

We estimated that the cost of analysing one sample was reduced by three since the derivatisation was suppressed, the procedure shortened and the mobile phase used at a very low flow-rate. We were able to determine a plasma concentration of busulfan in approximately 4.5 h compared to 11 and 17 h with the previous methods.

The HPLC–MS technique was used to determine the pharmacokinetic parameters of busulfan in 13 paediatric patients receiving the drug before bone marrow transplantation. In agreement with other published results for children, the pharmacokinetic parameters of busulfan were highly variable and differences related to age were observed. Younger children had higher oral clearances than older children [1,7]. The pharmacokinetic differences reported between adults and children [2] and between paediatric patients could be the result of differences in the bioavailability and/or in the metabolism of busulfan to γ-glutamyl-β-(*S*-tetrahydrothiophenium ion)-alanylglycine(THT^+) through conjugation with glutathione and catalysis by GSTA1-1 [9,27]. Using busulfan intravenously should reduce variability in drug distribution, but this remains to be evaluated.

Guided administration, using individual pharmacokinetic parameters such as mean concentrations or area under the plasma concentration–time curves should facilitate optimal dosage of most anticancer drugs [3,8]. Limited sampling strategies have been advocated to adjust the individual dosage of busulfan [10], but they may lead to errors in the determination of the AUC and in the predicted steady-state concentration [9]. Our method permits multiple small blood samples and is associated with an accurate and rapid determination of individual pharmacokinetic parameters.

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