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Quantification of busulfan in plasma by gas chromatography–mass spectrometry following derivatization with tetrafluorothiophenol

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Abstract

A specific and highly sensitive method has been developed for the determination of busulfan in plasma by gas chromatography–mass spectrometry using a deuterium-labeled busulfan (busulfan-d8) as internal standard. Plasma containing busulfan and busulfan-d8 were extracted with ethyl acetate and derivatized with 2,3,5,6-tetrafluorothiophenol prior to the monitoring of specific ions. The limit of quantification of the assay was 20 ng/ml and the calibration curve was linear over the range of 10 to 2000 ng/ml of derivatized busulfan. This method was in good agreement with the GC–MS assay using derivatization with sodium iodide and measuring diiodobutane. In addition, a pharmacokinetic study of busulfan was conducted in six children. The apparent oral clearance was 5.7±1.9 ml/kg/min and the volume of distribution was 1.0±0.4 l/kg and were similar to those previously reported in pediatric patients. © 1998 Elsevier Science B.V.

Keywords: Busulfan; Tetrafluorothiophenol

1. Introduction

Busulfan (1,4-butanediol dimethanesulfonate) (Fig. 1A), a bifunctional alkylating agent is currently administered in preparative regimens for bone marrow transplantation for patients with haematological malignancies and nonmalignant disorders such as thalassemia [1–3]. Busulfan is administered orally and the variability in its pharmacokinetics, including bioavailability, is important in children [4]. Therefore, drug monitoring and individual dose adjustment, performed with the aim of reducing graft rejection and severe toxic effects such as convulsions and veno-occlusive diseases [5–9] have been extensively discussed in the literature.

Several methods have been developed for the determination of busulfan in plasma, including gas chromatography with electron-capture detection (GC–ECD) [10–13], gas chromatography–mass spectrometry (GC–MS) [14,15], high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [16–18], and high-performance liquid chromatography–mass spectrometry (HPLC–MS) with a particle beam interface [19]. The sensitivity

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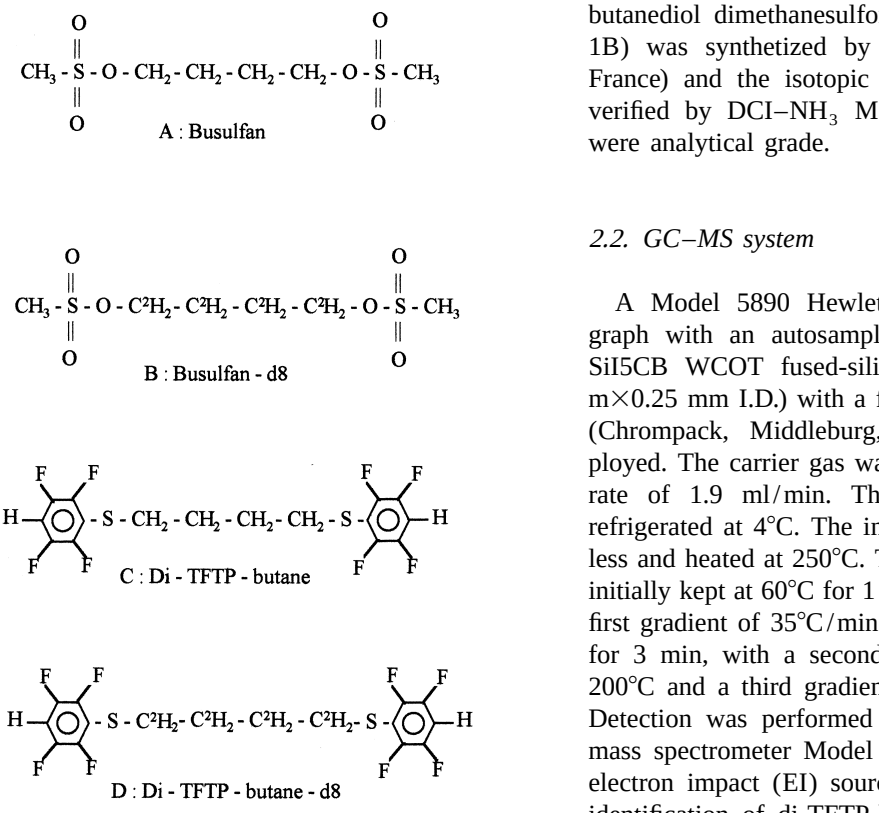

Fig. 1. Chemical structure of busulfan (A), busulfan-d8 (B), di-TFTP-butane (C) and di-TFTP-butane-d8 (D).

and reproducibility of the assay and the stability of the derivatives depend on the derivatization method selected [20]. All these methods but HPLC–MS require derivatization procedures. In this paper we report a new sensitive GC–MS method using derivatization with tetrafluorothiophenol and compare it with the previously published GC–MS assay using derivatization with sodium iodide [15].

2. Experimental

2.1. Reagents

Busulfan and 2,3,5,6-tetrafluorothiophenol (TFTP) were obtained from Aldrich (Saint Quentin Fallavier, France). The stable isotope [1,1,2,2,3,3,4,4-^{2}H]-1,4-butanediol dimethanesulfonate or busulfan-d8 (Fig. 1B) was synthetized by Eurisotop (Saint Aubin, France) and the isotopic enrichment (97.5%) was verified by DCI–NH_3 MS. All the solvents used were analytical grade.

2.2. GC–MS system

A Model 5890 Hewlett-Packard gas chromatograph with an autosampler, equipped with a CP SiI5CB WCOT fused-silica capillary column (25 m×0.25 mm I.D.) with a film thickness of 0.12 μm (Chrompack, Middleburg, Netherlands) was employed. The carrier gas was helium, used at a flow-rate of 1.9 ml/min. The autosampler tray was refrigerated at 4°C. The injector was in mode splitless and heated at 250°C. The oven temperature was initially kept at 60°C for 1 min then increased with a first gradient of 35°C/min to 170°C and maintained for 3 min, with a second gradient of 4°C/min to 200°C and a third gradient of 35°C/min to 250°C. Detection was performed using a Hewlett-Packard mass spectrometer Model 5971A equipped with an electron impact (EI) source. For the mass spectral identification of di-TFTP-butane (Fig. 1C) and di-TFTP-butane-d8 (Fig. 1D), a chemical ionization (CI) source was employed with ammonia as reactant gas. The two sources were heated at 175°C.

2.3. Busulfan assay in plasma

Stock solutions of busulfan (500 μg/ml) and busulfan-d8 (50 μg/ml) were prepared in acetone and stored in 1-ml aliquots at −20°C. An extraction and derivatization procedure, lasting 3 to 4 h and modified from the procedure previously described [10] was used. Briefly, 1 ml of plasma containing 100 μl of internal standard (2.5 μg/ml in acetone) was extracted with 3 ml of ethylacetate. The organic layer was dried under nitrogen at 60°C. The residue was dissolved in a mixture of water (0.2 ml), 1.5 *M* TFTP in methanol (20 μl) and 1 *M* sodium hydroxide (20 μl) and heated at 70°C for 2 h. After derivatization, 3 ml of sodium hydroxide and 3 ml of ethyl acetate were added and the organic phase was dried under nitrogen. The residue was dissolved in

100 μl of ethyl acetate and a 1 μl aliquot was injected into the GC–MS system.

2.4. Pharmacokinetic study

Plasma from six pediatric patients from India (Christian Medical College Hospital) and France (Hôpital Robert Debré) were studied. These children received busulfan as part of conditioning regimen before bone marrow transplantation for thalassemia major or myelogenous leukemia. The pharmacokinetic study of busulfan was performed during the first administration of the drug at the oral dose of 1 mg/kg. Seven blood samples from each patients were collected on ice in heparinized tubes in the 6 h following drug intake. Blood was immediately centrifuged and plasma was kept at −20°C until analysis.

Pharmacokinetic parameters were calculated by a non-linear extended least square program using a commercially available program (SIPHAR, Société Simed, Paris, France). Data were best fitted by a monocompartimental model. The following parameters were obtained: the apparent oral clearance (Cl/F: ml/kg/min), where F is the bioavailability, the volume of distribution (Vd/F: l/kg), the elimination half-life ($T_{1/2}$: h). In addition, the concentration at steady-state (C_{ss}, ng/ml) was calculated from the following equation:

$$C_{ss} = AUC_{0-\infty}/\tau$$

where τ is the interval between two consecutive administrations, i.e., 6 h.

2.5. Statistical analysis

Results are expressed as mean and standard deviation (S.D.). The coefficient of variation (C.V.) was also calculated.

3. Results

3.1. Mass spectra analysis

The mass spectra of di-TFTP-butane, [1.4-bis-(2,3,5,6-tetrafluorothiophenyl)butane] and di-TFTP-butane-d8, [1.4-bis(2,3,5,6-tetrafluorothiophenyl)-butane-d8] obtained after CI (Fig. 2) and EI (Fig. 3) were analysed. With both methods, limited molecular ions at m/z 418 for di-TFTP-butane and m/z 426 for di-TFTP-butane-d8 were observed. The ammonia CI mass spectra showed $[M+NH_4]^+$ adduct ions formation at m/z 436 for di-TFTP-butane and m/z 444 for di-TFTP-butane-d8 corroborating the molecular mass of these two compounds.

The CI exhibited predominant fragment ions at m/z 237 and m/z 245 corresponding to the loss of one tetrafluorophenol atom from di-TFTP-butane (m/z 237) and di-TFTP-butane-d8 (m/z 245), respectively. In contrast, the predominant fragment ions detected in EI were at m/z 195 and m/z 197. These ions, corresponding to a tetrafluorothiophenylmethane atom and its deutered analogue were not used for the assay because the ratio of m/z 195 over m/z 197 would be underestimated due to the presence of 4.2% of the natural isotope S34. Therefore, the monitored ions were focused at m/z 237 for di-TFTP-butane and at m/z 245 for di-TFTP-butane-d8.

3.2. GC–MS analysis of di-TFTP-butane and di-TFTP-butane-d8

Under the chromatographic conditions described, the retention time for di-TFTP-butane and di-TFTP-butane-d8 were 12.0 and 11.9 min (±0.8 min), respectively, without interfering peaks. Representative chromatograms obtained from a patient (A and B) and from a pool plasma (C and D) are shown in Fig. 4.

Sample recoveries from extraction measured at 250 ng/ml busulfan and 250 mg/ml busulfan-d8 were respectively, 78 and 84% for di-TFTP-butane and di-TFTP-butane-d8, respectively ($n=10$).

The sensitivity of the assay, at a signal-to-noise ratio of 3, was 10 ng/ml. The limit of quantification of the assay was 20 ng/ml.

The standard line plot of peak area ratio of di-TFTP-butane over di-TFTP-butane-d8 versus the corresponding standard concentration ratio of busulfan over busulfan-d8 was linear over the concentration range of 10 to 2000 ng/ml. The equation was: $Y=1.09X+0.0753$ ($n=5$). The C.V. of the slope of the calibration curves was 1.85% ($n=5$).

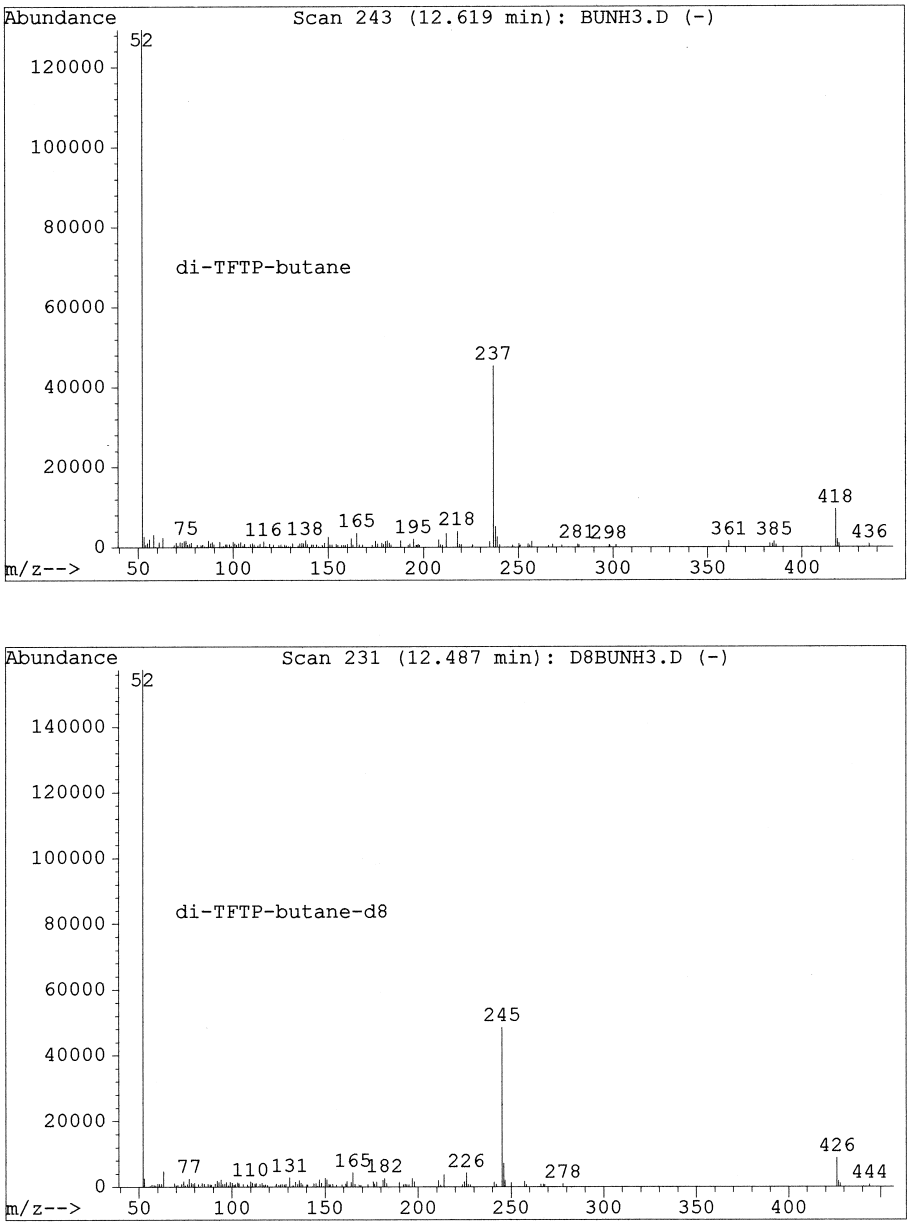

Fig. 2. Mass spectra of di-TFTP butane and di-TFTP-butane-d8 after CI.

Intra- and inter-assay coefficients of variation were determined from the calibration standards and from two quality controls (400 and 700 ng/ml busulfan) and are presented in Table 2. Intra-assay C.V.s were less than 10% except in calibration samples containing 10 to 50 ng/ml of busulfan. The accuracy evaluated by the recovery (ratio of di-TFTP-butane over busulfan content) is also presented. The intra- and inter-assay recoveries were between 90 and 100% for calibration standards and quality controls.

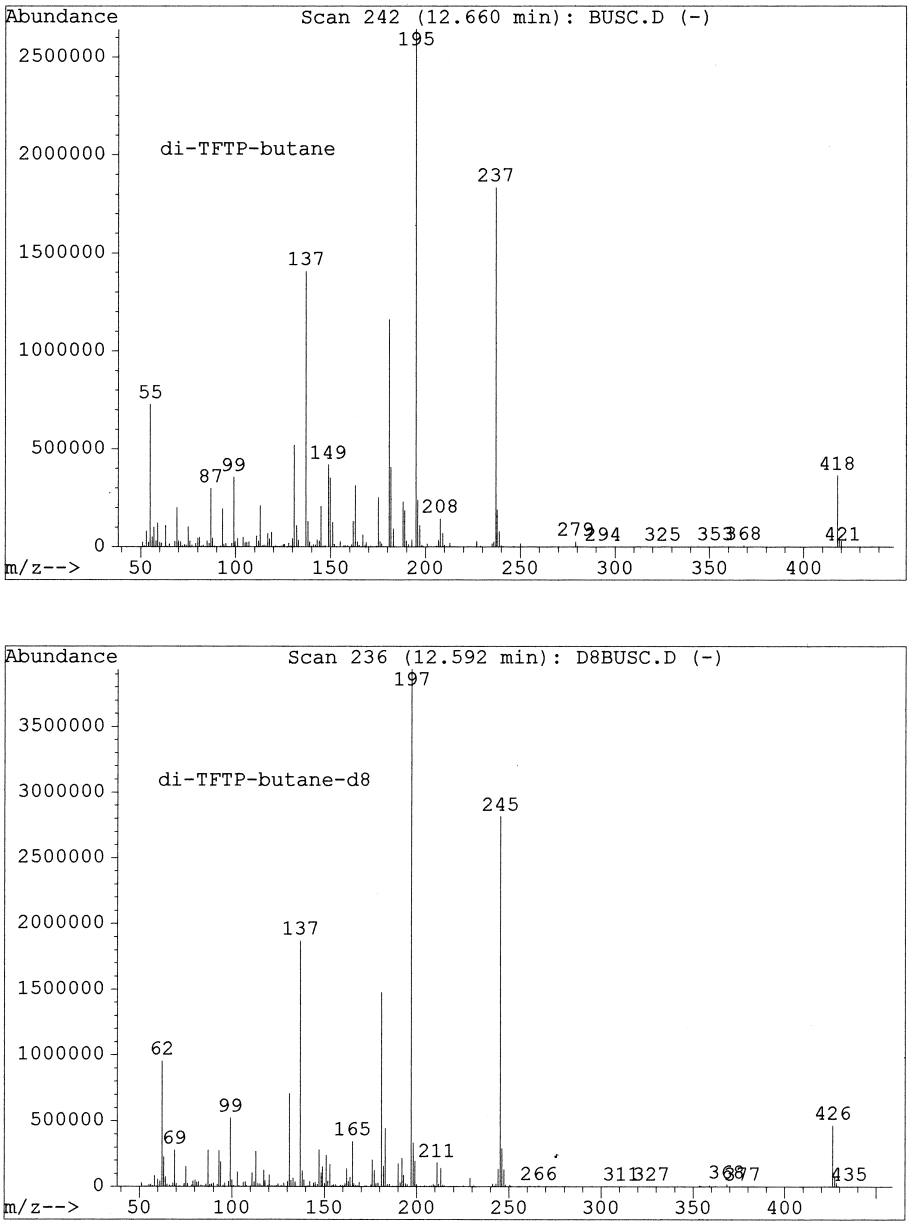

Fig. 3. Mass spectra of di-TFTP butane and di-TFTP-butane-d8 after EI.

Busulfan and busulfan-d8 in acetone were stable in sealed vials for one year at −20°C. The derivatized compounds in ethyl acetate were stable for 10 h at 4°C. The dried derivatives were stable for 24 h at −20°C.

3.3. Comparison with the GC–MS assay using derivatization with sodium iodide

This new method was compared with the GC–MS method of Vassal et al. [15], using sodium iodide

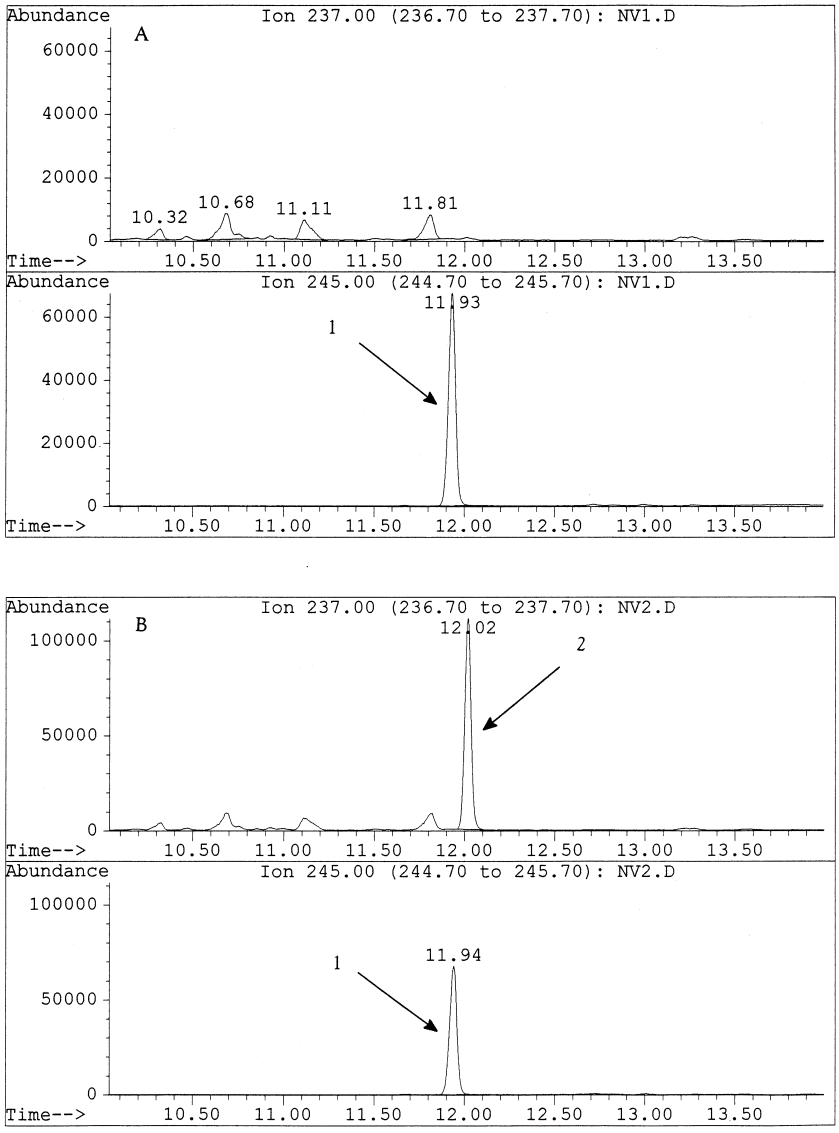

Fig. 4. Representative chromatograms obtained from a patient: (A) blank plasma spiked with 250 ng/ml busulfan-d8; (B) plasma obtained 30 min after the oral administration of 1 mg/kg and containing 270 ng/ml busulfan. Representative chromatograms obtained from a pool plasma; (C) double blank plasma; (D) plasma spiked with 10 ng/ml busulfan and 250 ng/ml busulfan-d8, peak 1 = di-TFTP butane-d8, peak 2 = di-TFTP butane.

derivatization. Briefly, plasma samples containing busulfan-d4 as internal standard were extracted in ethyl acetate, derivatized with sodium iodide (NaI), washed with water and the derivatized product, ie diiodobutane was injected into a GC–MS with single ion monitoring detection.

Nineteen samples, obtained from pediatric patients treated with busulfan were analyzed by both the two methods to compare the derivatization procedures using NaI or TFTP. The plasma concentrations ranged from 178 to 1264 ng/ml. Individual results compared by a Students' *t*-test for paired data were not statistically significant. In addition, the correlation between the diiodobutane and di-TFTP-butane

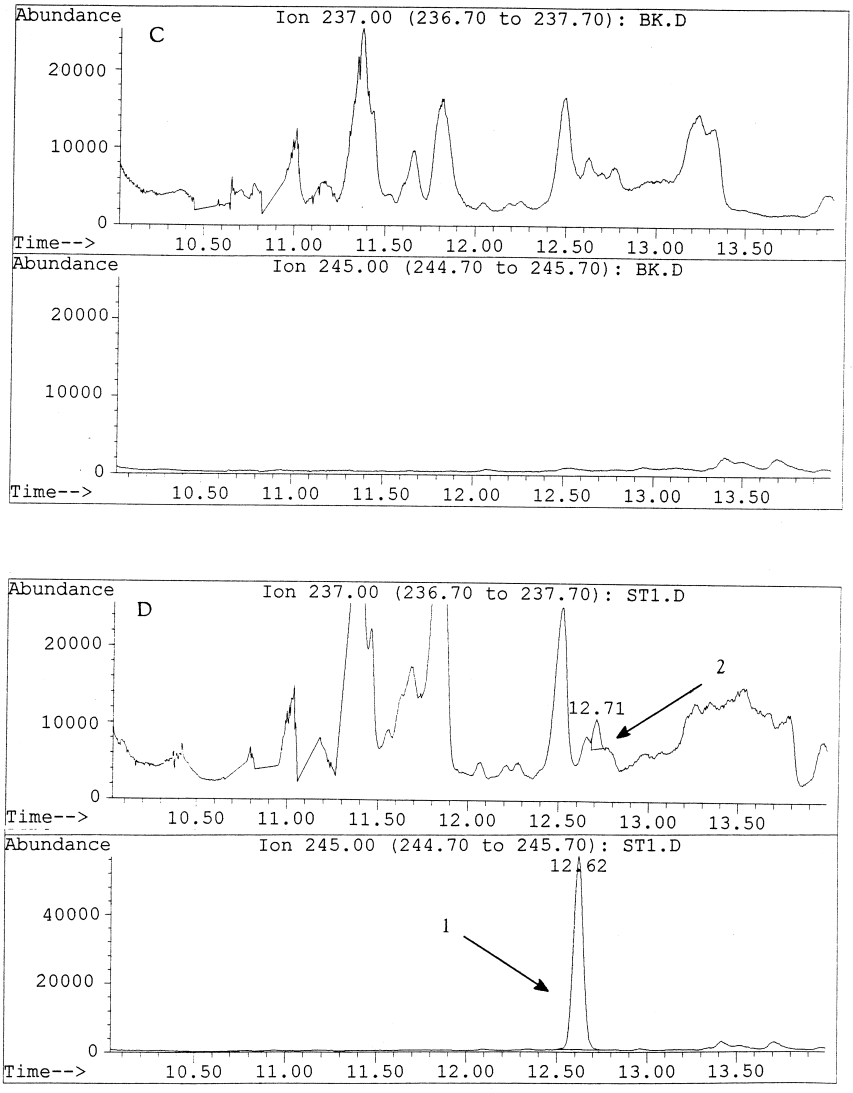

Fig. 4. (*continued*)

concentrations was highly significant ($p<0.001$) (Fig. 5).

3.4. Pharmacokinetic results

The individual pharmacokinetic parameters of the six patients, aged 5.8±5.0 years are presented in Table 1.

Mean Cl/*F* was 5.7±1.9 ml/kg/min (C.V., 33%), Vd/*F* was 1.0±0.4 l/kg (C.V., 40%) and $T_{1/2}$ was 2.0±0.3 h (C.V., 13%). The calculated individual C_{ss} values ranged from 328 to 761 ng/ml.

4. Discussion

Busulfan is a widely used antimitotic drug, only available as an oral preparation. The variability in drug absorption, associated with differences in drug disposition between adults and children [4] prompted us to develop a sensitive GC–MS assay to monitor

Table 1
Accuracy and precision of the method of quantification of busulfan in plasma by gas chromatography mass spectrometry following derivatization with tetrafluorothiophenol

Busulfan concentration (ng/ml)	Busulfan real content (ng/ml)	*n*		Mean concentration found (ng/ml)		C.V. (%)		Recovery (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Calibration standards									
0	6.25	4	4	6.1	5.6	64.3	62.0	97.0	89.3
10	16.25	4	4	16.4	15.0	24.4	22.9	101.2	92.4
20	26.25	4	4	25.7	24.6	25.2	12.3	98.1	93.8
50	56.25	4	4	55.5	53.4	13.5	4.9	98.6	94.9
100	106.25	4	4	94.1	94.9	8.1	3.7	88.5	89.3
250	256.25	4	4	244.8	254.1	3.8	5.0	95.5	99.2
500	506.25	4	4	497.2	497.6	5.7	1.9	98.2	98.3
1000	1006.25	4	4	981.8	968.9	3.8	3.3	97.6	96.3
2000	2006.25	4	4	2014.8	2015.1	0.7	0.7	100.4	100.4
QC samples									
400	406.25	4	4	403.8	407.3	3.0	1.4	99.4	100.2
700	706.25	4	4	700.0	702.3	0.7	1.0	99.1	99.4

C.V. = coefficient of variation (%).

plasma drug concentrations, in order to establish optimal doses and to avoid failures of treatment or toxicity in children receiving busulfan for bone marrow transplantation.

Among the published methods, the GC–MS methods, using a stable isotope as internal standard, either d4 [15] or d8, allow one to detect very low levels of the compound of interest with a very high specificity.

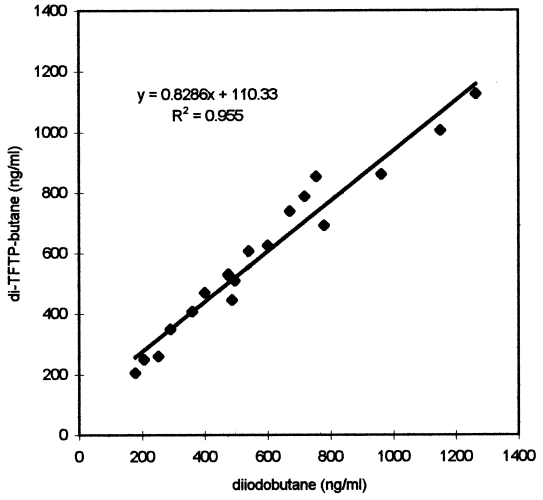

Fig. 5. Comparison of the concentrations of diiodobutane and di-TFTP-butane in 19 plasma samples.

The method of extraction and derivatization described in this paper is adapted from that of Chen et al. [10] and has numerous advantages: (1) the derivatization procedure is simple, performed in a single phase, avoiding the variability associated with stirring during NaI derivatization, (2) derivatized TFTP compounds are stable with time and resistant to heating, allowing the evaporation of the extraction phase without degradation, (3) the washing step with water is not necessary, (4) there is no interfering peak in plasma detected at m/z 237 and m/z 245. In addition, the concentrations of derivatized busulfan measured with the two derivatization procedures (TFTP and NaI) are in good agreement.

Busulfan disposition is known to be different in children compared to adults, with a higher clearance and larger volume of distribution [4]. The pharmacokinetic parameters that we determined are similar to those reported in pediatric patients, using either GC–MS [21–25] or GC–ECD [24–26] methods. After oral administration, busulfan is rapidly absorbed with a peak plasma concentration at 1 h. It is rapidly eliminated with an apparent oral clearance of 5.7 ml/kg/min. The elimination half-life is 2 h.

In conclusion, this new GC–MS method, based on TFTP derivatization is sensitive and easy to perform. It can be used routinely to monitor busulfan con-

Table 2
Pharmacokinetic parameters of busulfan after an oral dose of 1 mg/kg in six pediatric patients

Patient number	Age (years)	Weight (kg)	C_{max} (ng/ml)	T_{max} (h)	AUC (ng h/ml)	Cl/F (ml/kg/min)	Vd/F (l/kg)	$T_{1/2}$ (h)
1	3	12	895	1	3197	5.24	0.75	1.62
2	0.6	7.4	758	1	2933	6.14	1.09	1.94
3	0.7	8	578	1	2190	7.60	1.32	2.28
4	8.6	21	938	0.5	1970	8.00	1.56	2.26
5	11	25	1076	1.5	4568	3.50	0.63	2.07
6	11	31	1400	1	4402	3.70	0.57	1.80
Mean	5.8	17.4	941	1.0	3210	5.70	0.99	2.00
S.D.	5.0	9.7	282	0.3	1088	1.90	0.40	0.26
C.V. (%)	86	56	30	30	34	33	40	13

C_{max}: maximum concentration, T_{max}: time to reach the maximum concentration, $AUC_{0-\infty}$: area under the plasma concentration time curve extrapolated to infinity, F: bioavailability, Cl/F: apparent oral clearance corrected for bioavailability, Vd/F: volume of distribution corrected for bioavailability, $T_{1/2}$: elimination half life.

centrations in patients exposed to major toxic effects while receiving the drug.

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