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Monitoring of *N*,*N*-dimethylacetamide in children during i.v.-busulfan therapy by liquid chromatography–mass spectrometry

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Abstract

Recently, an intravenous (i.v.) formulation of busulfan using the potentially hepatotoxic and neurotoxic *N*,*N*-dimethylacetamide (DMA) as a solvent was introduced. There is a need to assess the exposure of DMA in patients during the intravenous high dose therapy. A rapid and selective LC–MS method was developed to quantify relevant DMA concentration in plasma. After protein precipitation with trichloroacetic acid, the isocratic separation was achieved using a 150 mm × 2 mm C_{18} column and elution with a mobile phase containing 0.1% formic acid in water/acetonitrile (97:3). Detection of DMA was carried out with a ThermoFinnigan single-quadrupole mass spectrometer in selected-ion monitoring mode as H^+-adduct at *m*/*z* 88.2. Deuterium-labelled DMA was used as the internal standard. The LC–MS method was accurate, precise and reproducible in the range from 0.25 to 150 mg/l and met the generally accepted criteria for bioanalytical methods. Two calibration ranges from 0.25 to 7.5 mg/l and 7.5 to 150 mg/l were used. The intra- ($n = 7$) and interassay ($n = 7$) accuracy and precision were both <7.7% and the limit of quantification is 0.25 mg/l. The method was successfully applied to investigate 203 plasma samples in children during the i.v.-busulfan therapy.

Keywords: *N*,*N*-Dimethylacetamide; LC–MS; Plasma; Busulfan

1. Introduction

N,*N*-Dimethylacetamide (DMA) is a water-miscible solvent being widely used for pharmaceutical and industrial chemical compounds with limited solubility in water. It has been employed in different intravenous pharmaceutical formulations such as in the intravenous formulation of busulfan [1,2].

The acute and subchronic toxicity of DMA has been studied in animals. The median lethal values ranged from 2.2 to 4.9 g/kg in mice and from 2.0 to 7.5 mg/kg in rats, depending on the route of administration. Toxicity concerns mainly the liver, central nervous system and skin [3–12]. Human data on the toxicity of DMA are limited. In 1962, DMA was studied as a potential anticancer drug [13]. The dose-related toxicities in that study were hepatotoxicity (i.e. increased liver transaminase levels) and neurological symptoms such as hallucination, somnolence, lethargy and confusion. Also, there were several trials of biological monitoring of workers exposed to DMA in the air [14–20].

Toxicological effects of DMA in pharmaceutical formulation are not investigated yet. As a consequence, the relative contribution of DMA to the hepatic and neurological toxicities observed in patients treated with the i.v. formulation of busulfan is difficult to ascertain. DMA itself may contribute to the overall toxicity of the formulation as patients receive high cumulative doses of DMA in combination with busulfan. There is a need to assess the exposure of DMA in patients during the high dose therapy. Therefore, a selective and rapid analytical method is required for the determination of DMA concentrations in plasma during the i.v.-busulfan therapy.

DMA and the metabolite *N*-methylacetamide (MMA) have been mainly detected in urine by gas chromatography coupled with flame ionisation detection, flame thermo-ionic detection or mass spectrometry [4,9,14,17,20]. In urine, MMA is a good marker for the DMA exposure. Only a few methods were developed to quantify DMA in plasma. Lindström et al. [21] published

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a gas chromatographic–mass spectrometric (GC–MS) method for the quantification of DMA in rats exposed to DMA in doses of 250 or 1000 mg/kg. In the literature, no method is reported to determine DMA using liquid chromatography–mass spectrometry (LC–MS). The availability of cheaper LC–MS instrumentation and the greater range of analytes suitable for this technique in comparison to GC–MS make LC–MS more attractive for bioanalytical laboratories in a clinical environment.

In the following, we describe a simple and selective LC–MS assay for the quantification of DMA in human plasma. The method is sufficiency sensitive to enable full pharmacokinetic profiling of DMA during the i.v.-busulfan therapy.

2. Materials and methods

2.1. Drugs and reagents

N,*N*-Dimethylacetamide (99.9%) was purchased from Aldrich (Steinheim, Germany). The internal standard (ISTD), $^{2}[H]_{9}$-*N*,*N*-dimethylacetamide (99%), was obtained from Deutero GmbH (Kastellaun, Germany). Other materials were obtained from the following sources: acetonitrile (HPLC-grade) from Roth (Karlsruhe, Germany), water (HPLC-grade) from J.T. Baker (Deventer, Netherlands), formic acid (analysis grade) and trichloroacetic acid (analysis grade) from Merck (Darmstadt, Germany) and liquid ammonia (28%) from Fluka Chemie (Buchs, Germany). Human plasma from healthy donors was supplied from the department of transfusion medicine, University of Münster.

2.2. Preparation of standard solutions

Stock solutions containing 10 g/l DMA and $^{2}[H]_{9}$-DMA were prepared in acetonitrile. Working solutions of DMA were diluted in water to obtain final concentrations of 1, 0.1 and 0.01 g/l. Two different solutions of $^{2}[H]_{9}$-DMA in water (10 and 25 mg/l) were used as internal standard. All stock solutions were stored at −20 °C. Plasma standards with concentrations between 0.25 and 150 mg/l were prepared by serial dilution of the working solution with sodiume citrate plasma to obtain the desired concentrations of DMA. Quality controls, containing 0.25, 1.0, 7.5, 25, 150 mg/l, were obtained by adding DMA working solution to 10 ml of blank plasma. The non-plasma spiking volumes of the quality controls are less than 15%. Aliquots were frozen in polypropylene glycol tubes at −80 °C and analysed together with patient's samples.

2.3. Liquid chromatography–mass spectrometry

Liquid chromatographic separation of DMA and $^{2}[H]_{9}$-DMA was performed with two L-7100 pumps and a L-7200 autosampler equipped with a column oven controlled by a D-7000 software (Merck-Hitachi, Darmstadt, Germany). An AQUA C_{18} analytical column (5 μm particle size; 150 × 2 mm I.D.; Phenomenex™) and a C_{18} guard column (4 mm × 2 mm I.D.) were applied. The mobile phase consisted of a mixture of water:acetonitrile (97:3) containing 0.1% formic acid using a flow-rate of 0.4 ml/min. Between runs, the column was rinsed with a mixture of water:acetonitrile (63:37) for 10 min and then re-equilibrated with the mobile phase for 6.5 min. A column-switching valve was used to switch the eluent into the detector 1.5 min after injection.

The mass spectrometric detection was carried out with a single-quadrupole mass spectrometer (Surveyor MSQ; ThermoFinnigan, Egelsbach, Germany) equipped with an electrospray source. Electrospray ionisation was performed using nitrogen as nebulizing gas at a flow rate of 11 l/min and 0.55 MPa nebulizing pressure. To optimize the ionisation of DMA and $^{2}[H]_{9}$-DMA, a solution of this compound in the mobile phase was injected without HPLC separation into the ion source. The MS operating conditions were set as follows: probe temperature 500 °C, corona voltage 3 kV and fragment voltage 50 V. Positive ionisation with selected-ion monitoring mode was used. DMA and $^{2}[H]_{9}$-DMA were detected as H^{+}-adduct at *m*/*z* 88.2 and 97.2 with a dwell time of 1 s.

2.3.1. Sample preparation

Two different procedures for sample preparation were used depending on the expected concentration:

(1) Dimethylacetamide between 0.25 and 7.5 mg/l:
Fifty microliters of plasma (patient plasma, calibration plasma or quality control samples) was diluted with 200 μl water and mixed with 10 μl of a solution of 10 mg/l of the internal standard. The sample was vortexed briefly, and subsequently deproteinated by addition of 25 μl trichloroacetic acid 50%. After vortex mixing for 30 s, the mixture was centrifuged at 10,392 × *g* for 8 min. Ten microlitres of liquid ammonia 7% was added to an aliquot of the aqueous supernatant (100 μl) and transferred into the autosampler vial. The injection volume was 10 μl.

(2) Dimetylacetamide between 7.5 and 150 mg/l:
Ten microliters of plasma (patient plasma, calibration plasma or quality control samples) was diluted with 1000 μl water and mixed with 10 μl of a solution of 25 mg/l of the internal standard. The sample was vortexed and deproteinated by addition of 100 μl trichloroacetic acid 50%. The further sample preparation was performed as described above.

2.3.2. Quantification

Two different calibration curves, ranging from 0.25 to 7.5 mg/l and 7.5 to 150 mg/l, were calculated by analysing six different standard solutions, respectively. Plasma samples with final concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 25.0, 50.0, 100.0, 150.0 mg/l DMA were prepared as described above. Calibration curves were obtained by plotting the peak area ratio *m*/*z* 88.2/97.2 of DMA and $^{2}[H]_{9}$-DMA against the initial DMA concentrations by least-square linear regression analysis with a weighting factor of 1/*x*.

Within every series, three quality control samples were analysed (0.25, 1.0, 7.5 mg/l or 7.5, 25.0, 150 mg/l). Precision and accuracy was assessed by repeated analysis of quality control samples in one series (intra-day) and on subsequent days

(inter-day). The limit-of-quantification (LOQ) was determined by measuring the lowest standard at least six times and accepted if the coefficient of variation (CV) was less then 20%.

2.4. Patient samples

Patient samples from a pharmacokinetic trial to introduce the DMA-based i.v. formulation of busulfan (Busulfex^{TM}) into paediatrics [22] were analysed. Nineteen children, aged from 0.9 to 17.3 years (median: 4 years) were included receiving i.v.-busulfan in 15 doses every 6 h of 0.8 mg/kg, 1.0 mg/kg or 30 mg/m^2 busulfan per administration containing overall DMA amounts between 437 and 6142 mg per dose. The first infusion was applied as a double dose over 4 h followed by the 2nd infusion 12 h later (instead of 6 h).

Blood samples were collected from the central venous line at 3 and 4 h (end of the infusion), 4.5, 5, 6, 8 and 12 h after beginning of the first infusion at day 1 of treatment and trough values prior to 4th and 8th dose. Optionally, blood samples were drawn 0 h (prior to infusion), 2, 3, 4, and 6 h after starting of the 13th, 14th or 15th dose.

The samples were drawn with ammonium heparinized tubes and centrifuged at 8 °C to separate the plasma fraction. Samples were stored at −80 °C until analysis.

3. Results

3.1. Liquid chromatography–mass spectrometry

The possibility of using electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) sources under positive ion detection mode was evaluated during the early stage of method development. ESI spectra revealed higher signals for the protonated molecule of DMA at *m/z* 88.2 [M + H$^+$] and ^{2}[H]$_9$-DMA at *m/z* 97.2 [M + H$^+$]. Different mobile phases such as aqueous solution of acetonitrile and methanol in the presence of acetic acid, formic acid or ammonium acetate were tested to optimize the sensitivity of the ESI ionisation. The best sensitivity was observed with water/acetonitrile, containing 0.1% acetic acid as the mobile phase. Additional tuning of the MS parameters such as probe temperature, corona voltage and fragment voltage further improved the response for DMA in the positive ion mode. The full scan product ion spectra of DMA and the ISTD in plasma are shown in Fig. 1.

The isolation and separation of DMA with reversed-phase liquid chromatography is extremely challenging due to the polar and hydrophilic properties of this solvent. Retention of these types of polar analytes requires the use of mobile phases containing very low organic components. Under these aqueous conditions, conventional C_{18} stationary phases were not suitable. The AQUA C_{18} analytical column demonstrated a sufficient retention with a capacity factor of 2.1. In addition the column was compatible with the polar mobile phase containing only 3% acetonitrile.

Under the developed chromatographic conditions, the retention time for DMA and the ISTD were 3.4 and 3.3 min, respectively. Between runs, the column was rinsed with a mixture of water and acetonitrile (63/37), to ensure that all contaminants were washed from the column. Therefore, the overall run time for each sample was 20 min. Shorter rinsing times with higher ACN amounts gave no reproducible results. Using this proce-

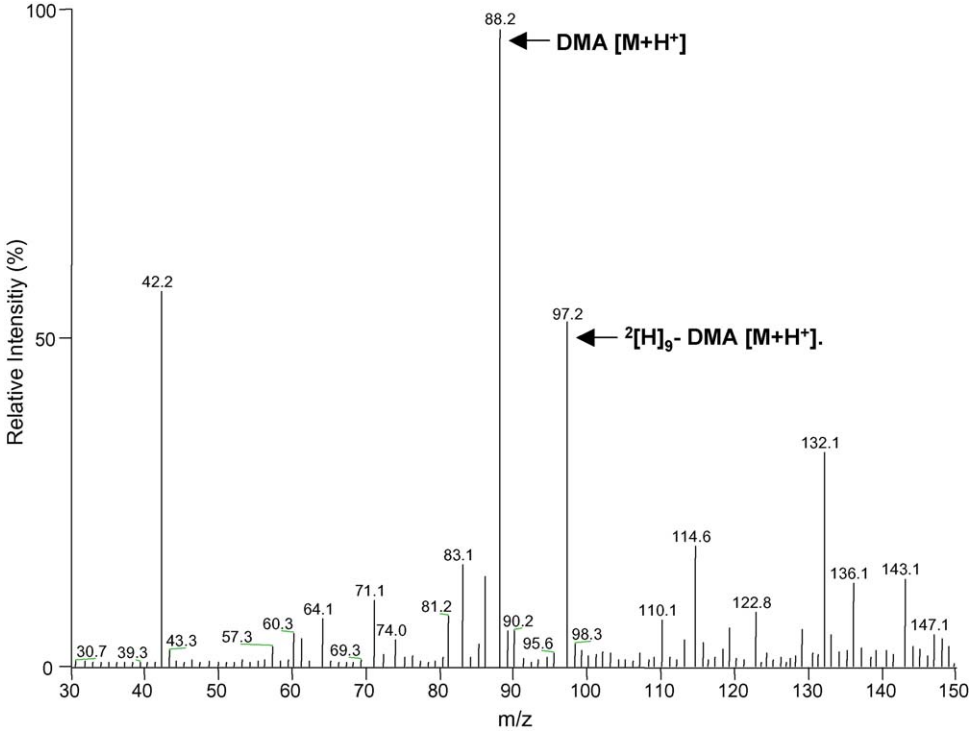

Fig. 1. Full scan spectra of [M + H$^+$]DMA and the ISTD ^{2}[H]$_9$-DMA.

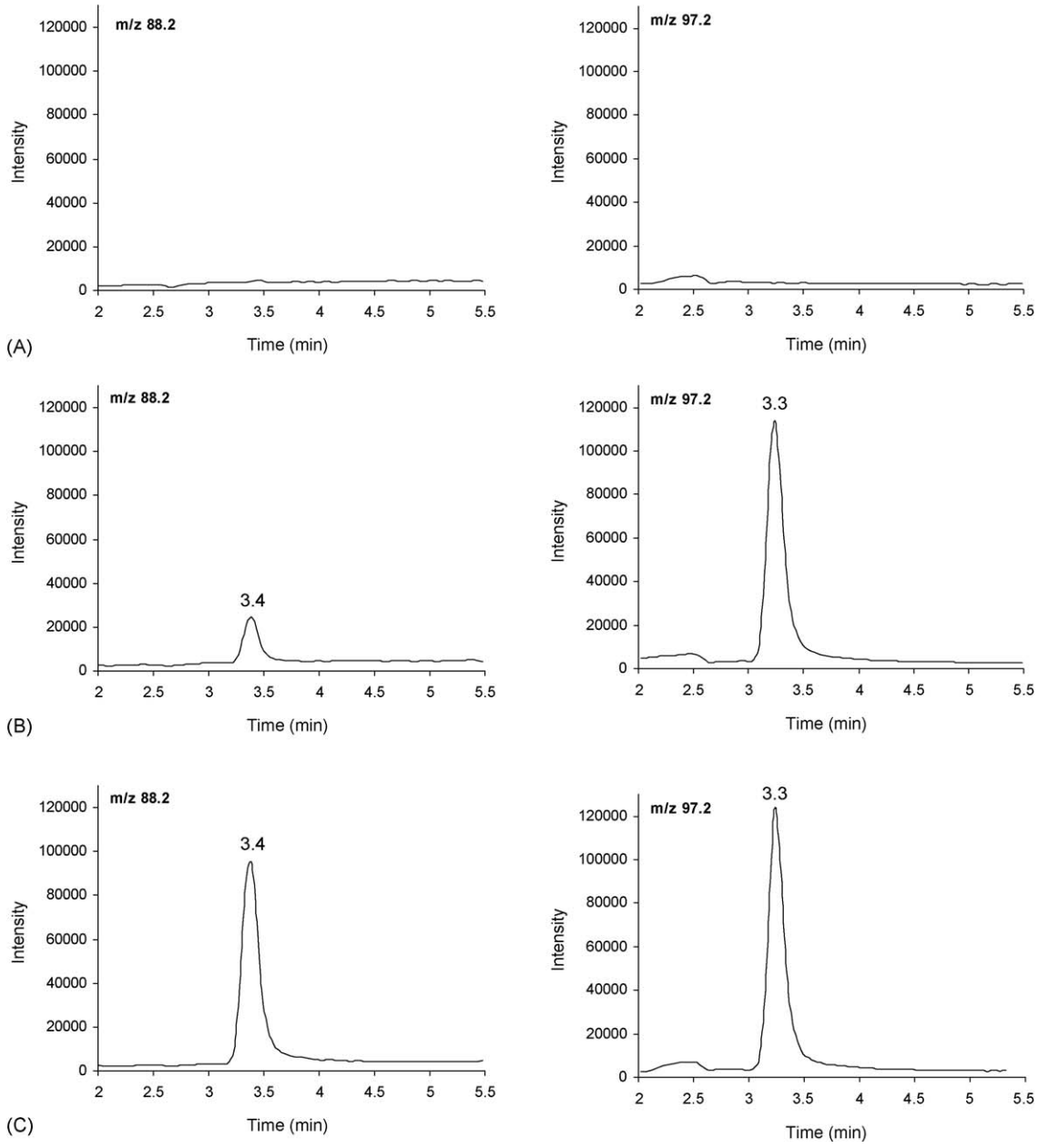

Fig. 2. Representative chromatograms from a blank plasma (A); spiked plasma with 0.25 mg/l DMA and $^{2}[H]_{9}$-DMA (B); and a patient plasma sample (C).

dure, there was no apparent shift in the retention times between the HPLC runs observed in the course of evaluation.

Deuterium-labelled DMA was used as the internal standard. The use of $^{2}[H]_{9}$-DMA with similar physical and chemical properties served to correct the deviations during sample preparation and in the detector response during quantitative analysis.

Chromatograms from a blank plasma samples, a quality-control sample at the limit of quantification (0.25 mg/l), and a plasma sample from one patient are shown in Fig. 2.

3.2. Sample preparation

The amphoteric character and the low volatility of DMA make it difficult to extract DMA from plasma by conventional liquid–liquid extraction. Different solvents (diethyl ether, chloroform, dichloromethane, *tert*-butylmethylether) were tested, but the recovery was unacceptable, possibly due to the volatility of the analyte. Thus, the plasma samples containing DMA were prepared by protein precipitation procedure [23,24]. Methanol, acetonitrile and trichloroacetic acid were investigated for precipitation. In the case of methanol and acetonitrile the resulting supernatant deteriorated chromatograms. The use of trichloroacetic acid resulted in sharp peaks and higher recovery. The use of trichloroacetic did not affect the chemical stability of DMA during sample preparation.

3.3. Method validation

Selectivity was assessed by comparing the chromatograms of six different blank plasma samples. No endogenous interfer-

Table 1
Accuracy and precision of the assay in one series (intra-day)

Added concentration (mg/l)	Mean calculated concentration (mg/l)	Accuracy (%)	Precision (%)	*n*
0.25–7.5				
0.25	0.26	102.3	2.0	7
1	1.03	103.4	2.7	7
7.5	7.48	99.7	4.5	7
7.5–150				
7.5	7.57	100.9	5.2	7
25	26.7	106.7	2.4	7
150	152	101.6	4.6	7

Table 2
Accuracy and precision of the assay on consecutive days (inter-day)

Added concentration (mg/l)	Mean calculated concentration (mg/l)	Accuracy (%)	Precision (%)	*n*
0.25–7.5				
0.25	0.23	92.3	6.0	7
1	1.04	103.9	2.4	7
7.5	7.42	99.0	3.5	7
7.5–150				
7.5	7.35	98.0	6.3	7
25	26.4	105.5	2.4	7
150	151	100.9	4.9	7

ences from human plasma were observed at the retention time of DMA and the ISTD.

The calibration curve was divided in two different ranges. Using a single calibration from 7.5 and 150 mg/l the precision and accuracy was not sufficient. With the sample preparation used in the lower range the detector response was not linear at higher concentrations. After particular dilution, the two calibration curves were linear over the concentration ranges 0.25–7.5 mg/l and 7.5–150 mg/l, with correlation coefficients >0.99. For the range from 0.25 to 7.5 mg/l the mean (±S.D.) *y*-intercept was 0.0376 (±0.0118), and the mean (±S.D.) slope was 0.0406 (±0.0041) over seven independent assay days. For the range from 7.5 to 150 mg/l the mean (±S.D.) *y*-intercept was 0.0304 (±0.0089), and the mean (±S.D.) slope was 0.5434 (±0.0082).

The intra-day and inter-day precision and accuracy were determined by analysing quality control samples at various concentrations as described in the experimental section. The intra-day precision (%CV) was found to be <5.2% ($n = 7$) for DMA and accuracy ranged from 99.7 to 106.7%. The intra-day precision and accuracy values are shown in Table 1. The inter-day precision (%CV) was found to be <6.3% ($n = 7$) and accuracy ranged from 92.3 to 105.5%. The inter-day precision and accuracy values are shown in Table 2. It is apparent that the method meets the generally accepted requirements for bioanalytical methods in terms of precision and accuracy [25,26]. Using a sample volume of 50 μl, the limit of quantification was found to be 0.25 mg/l.

3.4. Application

The developed LC–MS method has been used successfully to quantify DMA in human plasma following i.v.-busulfan administration. Overall, 204 plasma samples from 19 children were investigated. The absolute DMA amounts per infusion ranged between 437 and 6142 mg, depending on the applied dose of busulfan. The assay allowed the quantification of DMA in plasma down to the trough levels. The measured DMA plasma

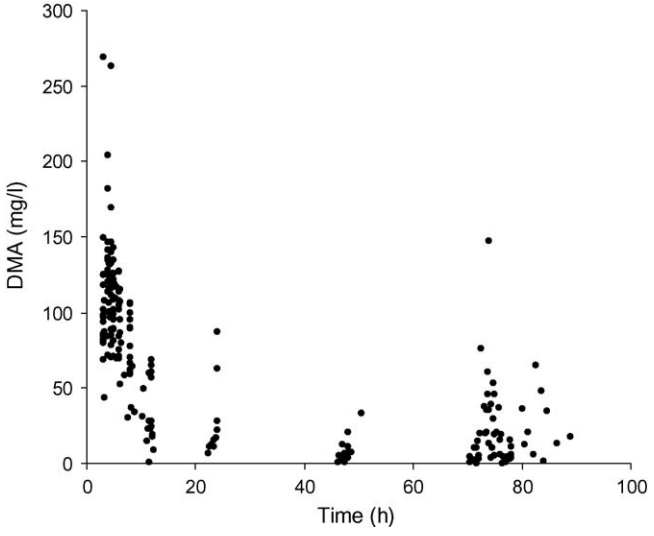

Fig. 3. DMA plasma concentrations in 19 children during the 4-day i.v.-busulfan therapy.

concentrations ranged from 0.34 to 149 mg/l and are shown in Fig. 3. The results from six samples are not shown because the concentration was not within the calibration range with one sample being lower than 0.25 mg/l and five samples higher than 150 mg/l.

4. Discussion

The quantification of DMA in biological material is problematic, due to the low-molecular weight, the missing chromophore and the polar character of DMA. Thus, previous investigations used gas chromatography due to its low boiling point. The detection of DMA with a UV detector is not possible. Consequently, DMA has been detected in urine by gas chromatography coupled to different detection systems. The published methods were used to analyse urine samples from workers occupationally exposed to DMA. Lindström et al. [21] published the only method suitable for plasma using GC–MS.

Since DMA is a small, organic molecule with high polarity, it is difficult to separate DMA by reversed-phase liquid chromatography. Firstly, we had to utilize a simple liquid chromatographic separation method compatible with the MS detection system.

Because the analysis has to be done in the low mass region, it was not surprising that the sensitivity of this method is relatively low. In this region, there is always considerable background noise due to fragments generated from the mobile phase or matrix compounds. Nevertheless, the sensitivity achieved is high enough to detect DMA plasma concentrations in all samples available. The DMA plasma concentrations during the intravenous busulfan were even so high, that some samples had to be diluted.

Using the described procedure with several dilution steps, the limit of quantification is 0.25 mg/l. Without dilution a lower limit of quantification could be achieved. However, in comparison with the older GC–MS method from Lindström et al. [21] the current method is more sensitive and it requires a smaller plasma volume of 10 or 50 μl plasma.

The limit of quantification achieved makes the assay appropriate for measurement of relevant DMA plasma concentrations during the i.v.-busulfan therapy. It is particularly useful for the pharmacokinetic analysis of DMA in children, where the sample volume must be kept to a minimum. Without the dilution steps, the method could also be optimized for a higher sensitivity which might be necessary in environmental investigations. Our results show that LC–MS can be used for the quantification of low-molecular weight liquids in biological matrices.

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