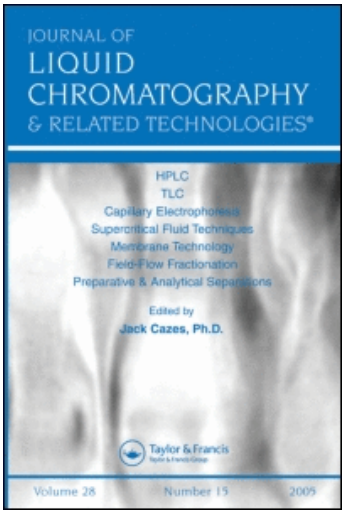

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Simultaneous Determination of Busulphan in Plasma Samples by Liquid Chromatography-electrospray Ionization Mass Spectrometry Utilizing Microextraction in Packed Syringe (MEPS) as On-line Sample Preparation Method

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Abstract: Busulphan is an alkylating agent used in high doses as preparative regimen before stem cell transplantation (SCT). Busulphan has a narrow therapeutic window and under- or overdosing may have a fatal outcome for the patient. Therapeutic drug

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monitoring followed by dose adjustment is currently used to adjust the exposure to busulphan. This is an important issue to optimise and individualise high dose therapy with busulphan. However, this approach is limited to centres with experienced personal measuring busulphan plasma concentrations. An automated and easy-to-handle method for measurement of busulphan plasma concentrations may facilitate and wide-spread drug monitoring approach and thus improve the outcome of the patients undergoing SCT.

Microextraction in packed syringe (MEPS) in combination with liquid chromatography and electrospray ionization mass spectrometric detection (LC-MS/LC-MS-MS) to quantify busulphan in human plasma samples without derivatization was developed. MEPS is a new miniaturised, solid-phase extraction technique that can be connected on-line to GC or LC without any modifications. In MEPS approximately 1 mg of the solid packing material is inserted into a syringe (100–250 μL) as a plug. The validation of the method showed that the selectivity, accuracy and precision for the method were satisfactory. This is well in line with the international criteria for the study validation. The present method has shortened extraction time considerably and the method is fully automated, which benefits therapeutic drug monitoring of busulphan in SCT.

Keywords: Busulfan, MEPS, LC-MS/MS, Pharmacokinetics, TDM

INTRODUCTION

Busulphan (Figure 1) is an anti cancer drug that is mainly used in high doses as preparative regimen before stem cell transplantation (SCT). High-dose therapy with busulphan (Bu) has been shown to be correlated to transplantation-related toxicities, such as veno-occlusive disease (VOD), interstitial pneumonia or neurotoxicity.[1,2] Pharmacokinetics and pharmacodynamics of Bu has been intensively studied with aim to define the therapeutic window for myeloablative and antileukemic effects, and thresholds for unwanted adverse effects. Several investigations have shown a wide inter- and intra-patient variation in pharmacokinetics. The variation is due to bioavailability,

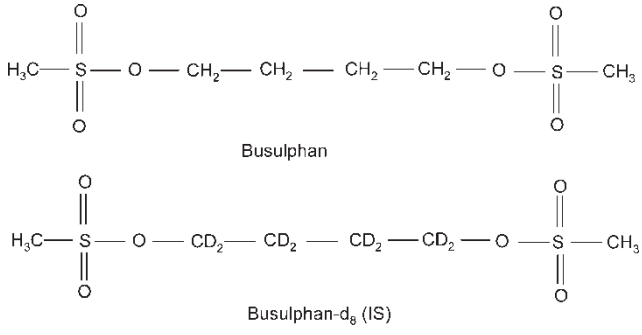

Figure 1. The structure of busulphsn and busulphan-d_8 (I.S).

age, underlying disease, drug-drug interaction and circadian rhythm.[3–9] The pharmacodynamic studies have shown a relationship between the exposure to busulphan and the clinical outcome for the transplanted patients. Low exposure to busulphan contributed to graft failure or relapse, and too high exposure to busulphan contributed to VOD and neurotoxicity.[10–14] Thus, therapeutic drug monitoring followed by dose adjustment to reach a target exposure is an approach how to optimise and individualise high dose therapy with busulphan.[13,15–17] However, this approach is limited to centres having possibility to measure busulphan plasma concentrations. The available standardised methods require experienced laboratory assistants and are time consuming. The determination of busulphan was performed by liquid and gas chromatography.[18–22] An automated and easy to handle method for measurement of busulphan plasma concentrations may facilitate and wide-spread drug monitoring approach and thus improve the outcome of the patients undergoing SCT.

Sample-preparation is an important part in the analysis of pharmaceutical compounds in body fluids. The aim of a sample-preparation is to eliminate interfering substances from the biological samples. Furthermore, the method should be reproducible, easy to handle, easy to automate with a minimum number of steps required.

Microextraction in packed syringe (MEPS) is a new technique for miniaturised solid-phase extraction that can be connected on-line to GC or LC without any modifications.[23–27] In MEPS, the solid packing material is inserted into a syringe as a plug. The plasma sample is drawn through the syringe by an autosampler to allow the analytes to be adsorbed to the solid phase. The solid phase is then washed by water to remove the proteins and other interfering material. The analytes are then eluted with an organic solvent or by the LC mobile phase directly into the injector. The process is fully automated. Any absorption material such silica based (C2, C8, C18), restricted access material (RAM) or molecular imprinted polymers (MIPs) can be used.

The MEPS technique differs from commercial solid-phase extraction (SPE) in that the packing is inserted directly into the syringe, not into a separate column. Thus, there is no need for a separate robot to apply the sample into the solid phase as with conventional SPE. The packed syringe can also be used several times, more than 100 times with plasma or urine samples and more than 400 times for water samples, whereas a conventional SPE column can only be used once. MEPS can handle small sample volumes (10 μL of plasma, urine or water) as well as large volumes (250 μL).

Compared with liquid-liquid extraction (LLE) and solid-phase extraction (SPE), MEPS will reduce sample preparation time and organic solvent consumption.

MEPS is fully automated and takes only about one minute for each sample.

The new technique can be used for complex matrices without problems which is not the case with SPME. Also, much higher extraction recovery can be obtained (60–90%) compared to SPME (1–10%).

The aim of the present investigation was to evaluate MEPS as a sample-preparation tool to determine busulphan in human plasma by LC-MS.

EXPERIMENTAL

Instrumentation

The high performance liquid chromatography (HPLC) instrument included two pumps, Shimadzu (Kyoto, Japan), an autosampler, CTC-Pal, (CTC Analytics AG, Zwingen, Switzerland) and a 20 μL sample loop. A Hypersil Gold (100 × 2.1 mm, 3 μm) column obtained from Thermo Electron Corporation (PA., USA) was used as analytical column. A Valco C4W valve, Valco Instruments (Houston, USA) was used as gate valve between the liquid chromatograph and the mass spectrometer. The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

A gradient HPLC was used with mixer volume of 0.1 mL. Mobile phase A was 100 mM ammonium formiat and acetonitrile 90:10 (v/v) and mobile phase B contained 100 mM ammonium formiat and acetonitrile 20:80 (v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then from 5 to 6 min isocratic at 80% of phase B and at 6.1 min phase B was set at 0% again. For system stability the next injection was performed after 9 min. The flow rate was 150 μL/min and sample volume (loading) was 25 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass QII Z-spray (Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode. The full scan mass spectra of busulphan and busulphan-d_8 (I.S) in ammonium acetate/acetonitril (1:1) solution showed ammonium-adducted molecules ($M + NH_4^+$) at *m/z:* 264 and 272 for busulphan and busulphan-d_8, respectively. For LC-MS the analysis were performed using selected ion monitoring (SIM). Also, multiple reaction monitoring (MRM) using precursor ion at *m/z:* 264 and 272 ($M + NH_4^+$) and after collisional dissociation the product ions 151 and 159 were used for quantification of busulphan and the internal standard, respectively (Figure 2). The optimum mass spectrometric parameter settings were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150°C and 300°C, respectively. Nitrogen was used both as drying (400 L h^{-1}), and nebulizing gases (20 L h^{-1}), the vacuum was $2 \cdot 10^{-5}$ in the mass analyzer and $2 \cdot 10^{-3}$ in the collision cell. Argon was used as collision gas and collision

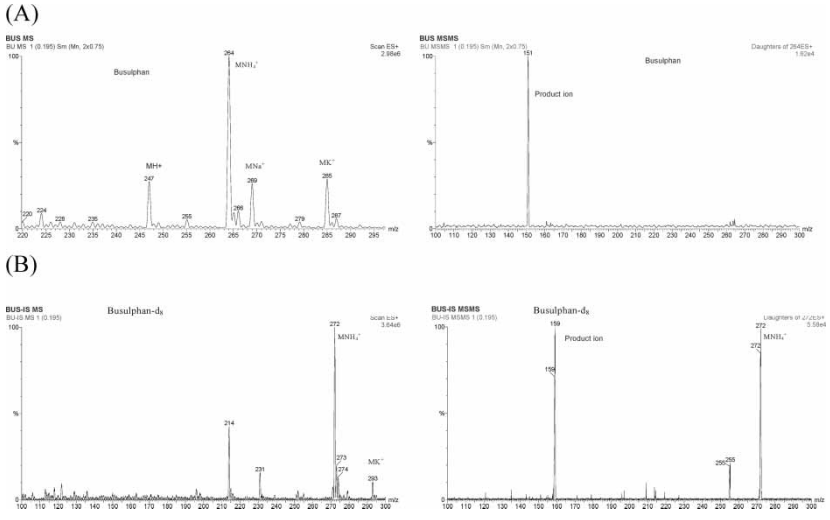

Figure 2. Mass spectrum of busulphsn and busulphan-d_8 for MS (A) and MS-MS (B).

energy was 25 eV. The gases were from AGA (Lidingö, Sweden). The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

Reagents and Materials

Busulphan was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Internal standard (busulphan-d_8) was synthesised as described previously.[20,28] Acetonitrile, methanol, formic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Preparation of Samples

Plasma samples were stored at -20°C. Before use, the plasma was thawed at room temperature and centrifuged at 3500 rpm for 10 min. Stock solutions of busulphan (10–100 μg/mL) and busulphan-d_8 (internal standard, 3 μg/mL) were dissolved in acetone. Spiked plasma samples were prepared by adding busulphan (10–50 μL) to 1.0 ml of centrifuged plasma. Twenty μL of busulphan-d_8 were added. After vortex mixing the samples were extracted and analyzed. The concentration range of the standard curve was between 5–2,500 ng/mL (5, 10, 50, 100, 200, 500, 800, 1000, and 2500 ng/mL, $\approx$20–10,000 nM). Quality control (QC) samples concentrations were 20, 400, and 1200 ng/mL. All standard and control solutions were stored at -20°C.

MEPS Procedure

MEPS was performed using a 250 μL gas-tight syringe. The sorbent used was a polystyrene polymer. This sorbent has irregular particles with average size of 50 μm and nominal 60 Å porosity. One milligram of the solid material was manually inserted inside the syringe as a plug. The sorbent material was tightened by filters to avoid moving inside the syringe.

Before using for the first time, the sorbent was manually conditioned with 50 μL methanol followed by 50 μL of water/methanol 90:10 (v/v). After that, the syringe was connected to the autosampler and the spiked plasma sample (50 μL) was withdrawn into the syringe by the autosampler. It is important that the plasma samples are withdrawn slowly (20 $\mu L\, s^{-1}$) and with caution to obtain good percolation between sample and solid support. The sorbent was then washed once with 100 μL of water/methanol 90:10 (v/v) to remove proteins and other interferences. The analytes were then desorbed by 25 μL methanol/water 95:5 (v/v) containing 0.25% ammonium hydroxide directly into a gate valve, which was situated between the liquid chromatograph and the tandem mass spectrometer. Cleaning of the sorbent was carried out using 5 × 50 μL elution solution followed by 5 × 50 μL of the washing solution between every extraction. This step decreased memory effects, but also functioned as conditioning step before the next extraction. The same packing bed was used for about 100–150 extractions before it was discarded.

Validation

Each calibration curve consisted of eleven calibration points covering from 5 ng/mL to 2500 ng/mL. Blank samples were run simultaneously. The plasma used was collected and pooled from different objects. The peak area ratios for busulphan and internal standard were measured and a standard curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

where y is peak area ratio, x is the concentration, a is the curvature, b is the slope and c is the intercept. The calibration curves were quadric and the weight was $1/x$. The QC samples were prepared with the concentrations of 20, 400 and, 1200 ng/mL. The accuracy and precision were calculated for the QC samples at three different assays. The method was validated at optimized conditions.

Accuracy was defined as the degree of deviation of the determined value and the nominal value: [(measure value-nominal value)/nominal value] × 100. Precision (C.V.%) was defined as the percentage of standard

deviation of the observed values divided by their mean values: [(standard deviation)/mean value] × 100.

RESULTS AND DISCUSSION

Method Development

The effect of different washing solutions on the recovery was investigated. The recovery was measured as the response of a processed spiked plasma sample expressed as peak area. The use of methanol in the washing mixture increased the leakage and decreased the recovery, however, clean extract was obtained. The lowest amount of leakage, with no interferences and the highest recovery was obtained with the use of 100 μL of water/methanol 90:10 (v/v) as washing solution.

To study the recovery, solutions containing methanol, water, formic acid and ammonium hydroxide were investigated as elution solutions. After introduction of the sample (50 μL) into the syringe and washing with 100 μL of water/methanol 90:10 (v/v), the elution efficiency was measured and compared to that of pure standard solution (1000 ng/mL). The eluting efficiency increased as the methanol content in the eluent increased. Acceptable recovery (≈60%) was obtained when using a solution of methanol/water 95:5 (v/v) containing 0.25% ammonium hydroxide and this was used as elution solution for validation of the method.

Method Validation

Selectivity

Plasma sample spiked with a mixture of busulphan and the internal standard were analysed and compared to blank plasma. No interfering compounds were detected at the retention times of the analytes using MEPS-LC-MS and MEPS-LC-MS-MS. Figure 3 shows patient plasma sample before and during the treatment. The results showed a good relationship between the concentration of busulphan and the response in the calibration range studied. The coefficients of determination (R^2) were >0.999 using plasma samples (Table 1).

Accuracy and Precision

The accuracy is determined as the ratio of the found and the theoretical concentration. The accuracy ranged from 97 to 107% (n = 18) for MEPS-LC-MS and from 98 to 107% (n = 18) using MEPS-LC-MS-MS (Table 2).

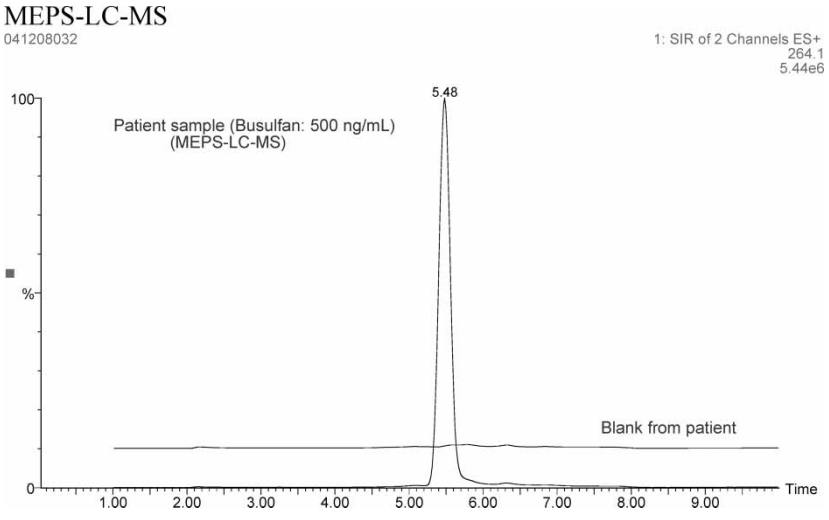

Figure 3. Mass chromatogram for patient blank plasma and patient plasma sample. Sample concentration: 500 ng/ml using MEPS-LC-MS.

The precision is determined as coefficient of variation of the within- and between-day variations at different concentrations. The intra-day precision was consistently between 1.6% and 2.3% for MEPS-LC-MS and from 2.2 to 3.8 for MEPS-LC-MS-MS. The inter-day variation of the precision was in the range of 6.4–9.2% and 6.4–11.3% for MEPS-LC-MS and MEPS-LC-MS-MS, respectively. The precision results are summarized in Table 2. The accuracy and the precision of the method were within the internationally accepted limits.[29]

MS, MS-MS Responses and Limit of Quantification

The LC-MS gave higher response (measured as peak areas) versus to LC-MS-MS. This it might be due to bad fragmentation of busulphan in the collision cell and the instability of the product ions obtained. The limits of

Table 1. Regression parameters for the calibration curve of busulphan in plasma

	Curvature (a) $\times 10^{-8}$		Slope (b)		Intercept (c)		R^2	
	MS	MS-MS	MS	MS-MS	MS	MS-MS	MS	MS-MS
Day 1	11.8	7.2	0.011	0.0017	0.001	0.001	0.9996	0.9998
Day 2	9.1	2.2	0.014	0.0019	0.002	0.005	0.9997	0.9999
Day 3	6.8	1.8	0.013	0.0017	0.002	−0.004	0.9998	0.9996

Table 2. Intra- and inter-day precision and accuracy for busulphan in plasma using MEPS-LC-MS/MS-MS

Concentration (ng/mL)	Accuracy		Precision			
	Accuracy (n = 18) (%)		Intra-day (n = 6) (R.S.D.%)		Inter-day (n = 18) (R.S.D.%)	
	MS	MS-MS	MS	MS-MS	MS	MS-MS
1200	99	98	2.3	3.3	7.0	6.4
400	107	107	1.6	3.8	6.4	7.3
20	97	106	2.1	2.2	9.2	11.3

quantification (LOQ) for busulphan were 5 ng/mL for LC-MS and 10 ng/mL for LC-MS-MS. At this concentration (5 ng/mL) the accuracy of LOQ was between 99% and 101% and the precision had a maximum deviation of 1% (n = 6).

Carry-over

Carry-over was tested by injecting blank plasma after the highest standard concentration. The carry-over was less than 0.1%.

Method Comparison (MEPS versus Protein Precipitation, PP)

Forty-seven plasma samples obtained from patients treated with busulphan were analysed using two different sample preparation methods MEPS and protein precipitation (PP) with LC-MS. Busulphan concentrations in plasma ranged from zero to 1600 ng/mL. There was a significant correlation between busulphan concentrations measured by LC-MS using MEPS and PP as sample preparation methods ($r^2 = 0.96$, $P < 0.0001$) (Figure 4). The variations between the two methods (MEPS and PP) were in the range of $\pm 15\%$ for the studied plasma samples. MEPS gave cleaner extract with lower noisy (Figure 3) compare to PP method (Figure 5). The signal-to-noise ratio (S/N) was improved by three fold using MEPS in comparison with PP as sample preparation method. Also, the backpressure in the LC-system increased using PP in comparison with MEPS.

Application of the Method

The method was applied for the analysis of plasma samples from different subjects and compared with a rutin metod utilizing gas chromatography-electron capture detection (GC-ECD) after derivatization of the busulphan. The deviation between the both methods was in the range of $\pm 15\%$.

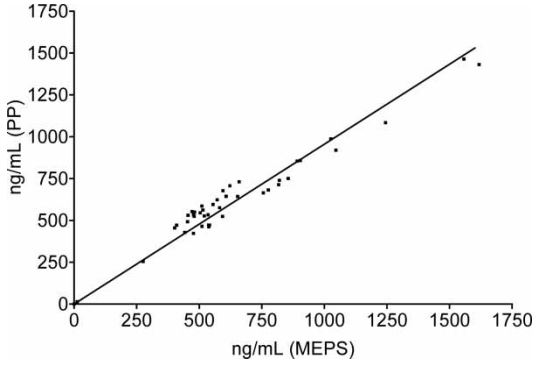

Figure 4. Correlation between the concentration of busulphan determined using MEPS-LC-MS and PP-LC-MS from forty seven patient plasma samples.

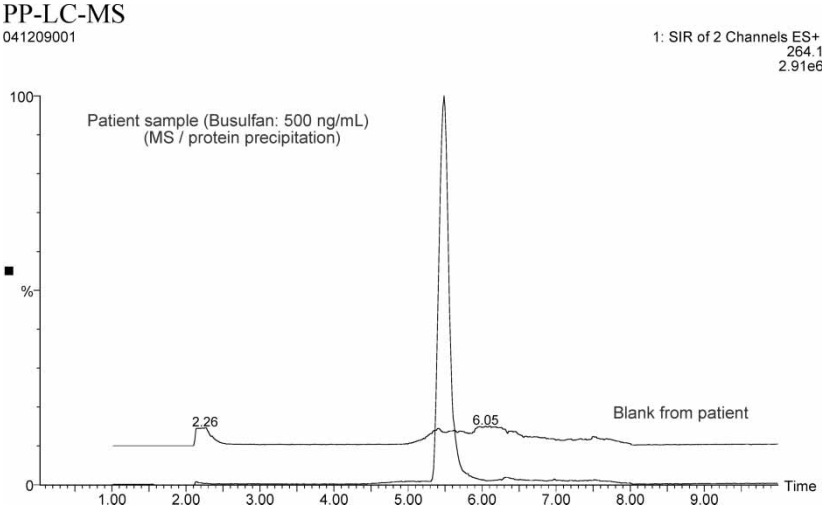

Figure 5. Mass chromatogram for patient blank plasma and patient plasma sample. Sample concentration: 500 ng/ml using PP-LC-MS.

Furthermore, the present method reduces the handling time for busulphan at least ten fold compared to earlier method.

CONCLUSION

Therapeutic drug monitoring (TDM) during high dos of busulphan has been reported to benefit the clinical outcome post stem cell transplantation by

reducing treatment related toxicity and minimize relapse rate. During the high dose treatment, patients receive busulphan dose every six hours, which requires a rapid and robust analytical method. The present method is fully automated and easy to handle. The results of accuracy and precision for busulphan obtained from the present study are comparable with these results reported in literature and are in good agreement with earlier published data. Apart from assay duration, accuracy and precision as well as extraction recovery must be considered when assessing the quality of a method. The present method provides both accuracy and precision within the range of therapeutic relevant levels (5–2500 ng/mL). Furthermore, the present method reduces the sample preparation time for busulphan (less than one minute per sample), which is of a great importance in adjusting busulphan dose in clinical settings.

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