Title

New Investigations into the Stability of Mesna using LC-MS/MS and NMR

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The above authors would like to confirm that there is no conflict of interest that we should disclose.

Abstract

It is important for sarcoma patients to receive the correct dose of Mesna as an adjuvant with Ifosfamide to reduce the risk of haemorrhagic cystitis. This paper describes a study conducted to evaluate the physicochemical stability of Mesna for injection formulation over 14 days.

Mesna samples (n=4, 20mg/mL) were incubated in glass vials at 37+0.5ºC. Mesna concentrations were determined by LC-MS/MS, and NMR was used to detect degradation products. Evaporative losses and pH were also monitored.

Our results differed from those published in existing literature. Both LC-MS/MS and NMR indicated that Mesna was unstable. The mean percentage decrease in Mesna concentration was 40% by day 14 of the analysis. The presence of Mesna's dimer Dimesna was detected on day 0 and its concentration increased over time. Dimesna was the only byproduct identified

To conclude, both LC-MS/MS and NMR analyses confirmed the instability of Mesna and its conversion into Dimesna.

Keywords: Mesna, Dimesna, Stability, Chemotherapy, Sarcoma.

Introduction

Mesna, sodium 2-sulfanylethanesulfonate, is a thiol-containing compound commonly used as an adjuvant, orally or intravenously with Ifosfamide and Cyclophosphamide, to reduce the risk of haemorrhagic cystitis caused by the by-product acrolein.(1–8)

Previous studies have confirmed that Mesna does not react with Ifosfamide, obstruct the tumour response to treatment or enter the cancer cells at all. This could be due to its hydrophilic properties, which prevents it from passing through the lipid biological membranes.(1,8–10) Mesna detoxifies acrolein via a similar mechanism to that of the naturally occurring thiol compound glutathione (found in the body in very low quantities).(1,11–13)

Upon entering the bloodstream, Mesna is oxidised to form its less active dimer Dimesna (dithio-*bis*-mercaptoethanesulphonate).(1,2) Similarly to Mesna, Dimesna is a hydrophilic compound which does not transfer across the lipid biological membrane through passive diffusion.(14) Upon reaching the kidneys, Dimesna is reduced back to Mesna and rapidly excreted due to its high solubility in water.(1,6) Previous studies have demonstrated that Mesna should be administered “continuously” to patients to prevent haemorrhagic cystitis.(15–22)

One of the regimens for the treatment of metastatic soft tissue sarcoma (MSTS) is the combination of Mesna with Ifosfamide (1:1) as a continuous intravenous infusion via elastomeric pumps for 14 days.(23) To ensure patients safety and reduce haemorrhagic cystitis, it is important to demonstrate that Mesna is stable and the desired dose is maintained for the duration of the infusion.

Previous work carried out by the authors of this paper on the stability of Ifosfamide and Mesna in elastomeric pumps using HPLC-PDA, showed an apparent increase in Mesna concentration

over the duration of analysis (24,25) though other investigators (using HPLC-UV) have indicated that Mesna was either stable or had a clinically insignificant decrease in its concentration over time. (26–28) However, other authors have suggested that HPLC with UV detection is not a suitable technique for the analysis of Mesna/Dimesna. James et al (1986) described the detection and quantification of Mesna and Dimesna in the patients' urine using high performance liquid chromatography coupled to electrochemical detection.(29) Verschraagen et al also used electrochemical detection to quantify Mesna and Dimesna concentrations in patients' plasma and urine.(30) Yet many subsequent studies have used HPLC-UV to investigate the stability of Mesna under various conditions.(26–28,31)

Despite the widespread clinical use of Mesna, few studies have investigated its stability using a suitable analytical method (other than HPLC-UV) and none considered the possibility of Dimesna forming in the infusion. Therefore, the aim of this study was to evaluate the physicochemical stability of Mesna and quantify any Dimesna forming at 37ºC for average adult doses; using a validated Liquid Chromatography-Mass Spectrometry (LC-MS/MS) method and Nuclear Magnetic Resonance Spectroscopy (NMR).

Materials and Methods

a. Materials

Mesna for injection formulation (400mg/4mL) was obtained from Baxter Healthcare Ltd (West Berkshire, UK). Dimesna 100mg was purchased from Lorne Laboratories (Houston, USA). Water for Injection was purchased from HameIn Pharmaceuticals (Gloucester, UK). HPLC grade water and acetonitrile were obtained from Fisher Scientific (Loughborough, UK). Analytical grade Mesna, sodium bicarbonate, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt, ethylenediaminetetraacetic acid (EDTA) and ethyl-4-hydroxybenzoate were purchased from Sigma Aldrich (Poole, UK).

b. Sample preparation

Mesna for injection samples (n=3, 20 mg/mL) were prepared by diluting Mesna for injection ampoules from two different batches (samples 1& 2 from batch 1A314, sample 3 from batch 2A051) with water for injection (**Table 1**). Samples were incubated in glass vials at 37±0.5ºC. For LC-MS/MS analysis, each sample was diluted further (0.0005 mg/mL) in triplicate using water for injection and 1000 µL of the 0.001 mg/mL internal standard solution. A separate Mesna sample (sample 4, batch 2A051) was prepared for the NMR analysis, deuterium oxide (10 % v/v, for 1 ml total volume) was added during the sample preparation and a trace amount of 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt was used as a reference.

c. Instrumentation

The LC-MS/MS analysis was performed using a Thermo system consisting of an Accela autosampler (Thermo Scientific, UK), Triple Quadrupole TSQ quantum access mass spectrometer (Thermo Electron Corporation, UK), an Accela gradient pump (Thermo Scientific, UK) and a reversed-phase C8 ultrasphere column (4.6 mm x 250 mm, 5 µm; Beckman Coulter

6UE0255). Ethyl-4-hydroxybenzoate was used as an internal standard. The column temperature was maintained at 37°C and the autosampler temperature was held at 20°C. The mobile phase was acetonitrile (Solvent A) and 1% aqueous ammonium bicarbonate (Solvent B). The flow rate was 1000 µL/min and the solvent composition was 50:50 for 3 minutes before Solvent A was increased linearly to 100% at 4.5 min. Electrospray ionisation (ESI) was used in negative ion mode with spray voltage of 4000 V and capillary temperature of 250°C. Detection was by selected reaction monitoring (SRM) with the ion transitions as shown in **Table 2**.

For NMR analysis, analytical grade Mesna and Dimesna (20 mg/mL) were initially run on a Brüker Avance III 400 MHz FT-NMR spectrometer using a gradient enhanced 1D NOESY water suppression pulse sequence to generate 1D proton spectra. TopSpin 3.0 pl4 and Icon NMR 4.5.4 build 51 control and processing software were used to acquire and process the data. Subsequently, Mesna for injection (20 mg/mL) was run on a Brüker Avance III 600 MHz FT-NMR spectrometer using the same experiment to obtain 1D proton spectra. The lower resolution spectrometer was equipped with a 5 mm broadband multinuclear probehead (PABBO BB-1H/D Z-GRD, Bruker BioSpin GmbH) whilst the higher resolution spectrometer was equipped with a 5 mm triple resonance inverse probehead (PATXI 1H/D-13C/15N Z-GRD, Bruker BioSpin GmbH).

d. LC-MS/MS method development and validation

Fresh samples of Mesna (1 mg/mL) were prepared each day and used to prepare 7 calibration standards that were analysed immediately. Dimesna stock solution (1 mg/mL) was prepared and stored at -80ºC to prepare 7 calibration standards daily. Furthermore, QC samples of Mesna were spiked with either HCl or EDTA to attempt to stabilise Mesna in solution. The LC-MS/MS method was tested for accuracy, precision, linearity, limit of detection (LOD) and limit of

quantification (LOQ) following both the International Conference on Harmonisation (ICH) and the Food and Drugs Administration (FDA) guidelines.(32,33)

Precision: Intraday precision, was determined by analysing 3 concentrations of Mesna in triplicate. Mean, standard deviation and the coefficient of variation were calculated.

Accuracy: Accuracy was determined by analysing 3 concentrations of Mesna in triplicate and comparing their measured concentrations to the known values.(34)

Calibration Curves: the instrument was calibrated in the range 0-1000 PPB.

Limits of Detection and Quantification: Limit of detection (LOD) and quantification (LOQ) determination were based on the standard deviation of the response and the slope approach.(32) Three replicates of Mesna were analysed and their standard deviation was calculated together with the slope of their calibration curves.(34,35)

e. Chemical and physical stability tests

The chemical stability of Mesna was assessed by LC-MS/MS, NMR, evaporative loss and pH analysis. The physical stability was also assessed by visual examination under normal fluorescent light for the presence of particulates and colour changes.

f. Samples and Data Analysis

Each vial was subsampled on days 0, 1, 4, 7, 8, 9, 11 and 14 of the experiment. Each subsample was diluted further using water for injection and ethyl-4-hydroxybenzoate (internal standard) producing 3 solutions that were analysed in triplicate. A further Mesna sample was analysed on a Bruker Avance III 600 MHz FT-NMR spectrometer. The water peak position, suppressed in the final 1D proton experiments, was determined each time using a single pulse experiment involving a 360deg pulse. The receiver gain was determined for the first experiment in the series

(at T0) and was kept constant throughout the study. Thus only O1P (the irradiation offset, set to the water peak position) was optimised each time (around 4.70ppm). The sweep width was 10.0 ppm (6.0 KHz) centered on the water peak. The spectra were recorded as the average of 16 scans of 65K complex data points. The data were processed using a single exponential windowing function (lb = 0.2 Hz) and the baseline was automatically adjusted in each spectrum using a polynomial (degree 5) function.

Results and Discussion

- **LC-MS/MS Method Validation**

Validation results for both compounds were within the limits specified by regulatory guidelines (**Table 3**).(33) Analysis of QC samples of Mesna was difficult due to the fast formation of Dimesna during sample preparation. Attempts to stabilise Mesna/Dimesna by the addition of EDTA and acid (HCl) were unsuccessful and affected the chromatography resulting in poor peak shapes and so were discontinued.

- **Chemical and Physical Stability of Mesna using LC-MS/MS and NMR**

The results obtained from LC-MS/MS analysis showed that Mesna was unstable over the duration of analysis. Dimesna was detected on day 0 and its concentration increased over time at 37°C. Mesna concentration dropped over the duration of the experiment (**Figure 1**). The mean percentage of loss in Mesna concentration for the three samples was 40% by day 7 and remained constant until day 14; statistical analysis confirmed the significance of the trends shown in **Figure 1** (ANOVA, $p<0.001$).

Mesna's dimer was either present initially in the vial or started forming immediately upon dilution, by the formation of a disulphide bridge between two Mesna monomers.(1) It was previously reported by Verchraagen et al that the stability of Mesna in urine samples was improved by increasing ethylenediaminetetraacetic acid (EDTA) concentration in the samples.(30) Verchraagen et al results demonstrated that the half-life of Mesna increased by up to 6 times in urine samples containing EDTA or hydrochloric acid. EDTA is added by the manufacturer to the pre-formulation vials to stablise Mesna.

The rate of auto-oxidation reaction and the amount of Dimesna formed was different for samples made from different batches of Mesna as shown in **Figure 2**. Samples 1 and 2 were prepared from the same batch and had a higher percentage of loss in Mesna concentration and Dimesna formation over time (64% and 49% respectively), while, sample 3 had only 34% loss in Mesna concentration by day 14. The manufacturer's tolerated concentration range for EDTA in the Mesna for injection formulation is between 0.2 - 0.3 mg/mL. This variation in EDTA concentration will affect the rate of dimer formation explaining the different rate of degradation between batches.

The NMR analysis of sample 4 corroborated the results obtained by LC-MS/MS. The NMR integral regions for Mesna and Dimesna were 3.37-3.32, 3.16-3.08 and 3.26-3.19, 3.07-3.02 ppm respectively. Dimesna was present in the solution from day 0 and increased in concentration over time as shown in **Figure 3** and **Figure 4**. Quantitative analysis of the ^{1}H NMR data has also confirmed the trend of Dimesna formation. In particular, it demonstrated a similar trend to that of Mesna sample 3 (both samples 3 and 4 are from the same batch). Mesna concentration decreased rapidly up to day 7 (**Figure 5** and **Figure 6**). Dimesna was the sole reaction product detected by

NMR. This finding was in agreement with the findings of James et al in a study that investigated the metabolism of Mesna in plasma.(36)

The pH of samples 1 - 3 was determined and found to be in the range 7.9-6.8 (average readings were 7.9 on day 0, 7.5 on day 4, 6.8 on day 14). This drop in pH would be a consequence of the auto-oxidation of Mesna mechanism of reaction, which releases protons during Dimesna formation (**Figure 7**). Evaporative losses were insignificant, below 1% over the duration of the experiment.

The physical stability results showed that the Mesna solutions remained clear and colourless until day 4. White solid particles were observed by day 7 in all three samples. Mesna is highly soluble in water (>100 mg/mL) and therefore it is unlikely that the particles are Mesna crystals. The drop in the pH of the solution may promote Dimesna precipitation.

Expert Commentary

In conclusion, a reliable and specific LC-MS/MS method was developed for the detection and quantification of Mesna and Dimesna. The Triple Quadrupole Mass Spectrometer is a far more selective detector than the PDA used previously in existing published methods. Both LC-MS/MS and NMR analyses confirmed the instability of Mesna and its conversion into Dimesna. The mean percentage decrease in Mesna concentration was 41% at 37°C by day 14. Dimesna was found to be the only degradation product formed and the extent of conversion varied among the Mesna samples possibly due to batch variations in EDTA concentration.

Overall, the findings of this experiment were different to those of previous studies (26,27,37) and showed that Mesna was not stable, forming Dimesna. The use of Dimesna was tested in combination with Cisplatin and Paclitaxel or Docetaxel during clinical trials phase I and II to

treat patients suffering ovarian, lung and head and neck cancer.(38,39) Recently, Dimesna was also used for clinical trials phase III as a continuous intravenous infusion along with Taxane and Cisplatin chemotherapy for the treatment of non-small lung cancer, however it is not a licensed drug.(40)

Clinically, Dimesna possesses similar therapeutic properties to Mesna when administered intravenously as Mesna dimerises immediately upon entering the bloodstream.(1,2) According to our results, treatments given to patients via elastomeric pumps will not only contain Mesna, but also Dimesna. However, the presence of Dimesna, and particularly the drop in pH associated with its formation may destabilise other drugs in the formulation causing sub therapeutic dosing and affecting treatment efficacy. Furthermore, the physical instability of the Mesna formulation, and the formation of particles by day 7 will affect the total Mesna dosage given to patients, and if this effect is significant, worsen the effects of haemorrhagic cystitis or delay the end of infusion by blocking the pumps' filter. Further studies would be needed to establish whether this has any clinical significance.

It is important to test the combination of Ifosfamide and other drugs administered with Mesna in order to determine synergistic effects that may affect the stability of the formulation and possible effects on treatment outcomes.

Five-year View

This paper showcased the stability profile of a common regimen, Ifosfamide/Mesna used for MSTS patients. It is interesting to note that all previous stability studies published on Mesna showed that it was chemically stable. The findings of this manuscript were different to those of previous studies as Mesna was shown to be not stable, forming Dimesna. This was not evident in the previous studies mainly because of the specificity/selectivity of the used analytical procedure - HPLC with UV detection - is not a suitable technique for the analysis of Mesna/Dimesna.

Results of this paper indicate the clear need to investigate the stability of anticancer therapies, under similar conditions to those used in practice, using a suitable and specific analytical method. It is anticipated that more stability tests should be performed on current chemotherapy regimens duplicating exact standard clinical practice conditions i.e. concentration of the drugs, type of delivery container, exact diluent, temperature and other incubation parameters. Each of these parameters might alter the stability of the drug of interest and lead to sub-dosing the patients, introduce additional side effects depending on the toxicity of the degradation products and/or affect the chemical compatibility within the formulation.

Key Issues

- Results detailed in this manuscript describe an example where ensuring the specificity/selectivity of an analytical procedure is important to avoid producing misleading results. HPLC with UV detection was shown to be a not suitable technique for the analysis of Mesna/Dimesna.

- The conversion of Mesna into Dimesna has not been reported before and implies that MTST patients prescribed with IV Ifosfamide/Mesna, over 7 days using elastomeric pumps are also receiving Dimesna in their infusion..

- Temperature is a key parameter to consider for the stability of anticancer therapies. Regulating the temperature surrounding the elastomeric pump, depending on the chemotherapeutic formulation and its stability profile / rate of degradation, may improve the stability of the therapy.

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Conflict of Interest

The Authors of this paper declare that there is no conflict of interest.

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