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Quantification of BNP7787 (dimesna) and its metabolite mesna in human plasma and urine by high-performance liquid chromatography with electrochemical detection

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Abstract

A sensitive and accurate assay was developed and validated to determine BNP7787 (dimesna), a new protector against cisplatin-induced toxicities, and its metabolite mesna in plasma and urine of patients. Both analytes were measured as mesna in deproteinized plasma or in urine diluted with mobile phase using high-performance liquid chromatography with an electrochemical detector provided with a wall-jet gold electrode. The assays for BNP7787 and mesna in deproteinized plasma were linear over the range of 1.6–500 μ*M* and 0.63–320 μ*M*, respectively. In plasma, the mean recovery of BNP7787 over the whole concentration range was 100.6% and of mesna 94.6%. The lower limits of quantitation (LLQs) of BNP7787 and mesna in deproteinized plasma were 1.6 μ*M* and 0.63 μ*M*, respectively. For both compounds the within- and between-day accuracy and precision of the assay was better than 12%. The assays for BNP7787 and mesna in urine were linear over the range of 0.8–1200 μ*M* and 0.63–250 μ*M*, respectively. In urine, the mean recovery of BNP7787 over the whole concentration range was 94.1% and of mesna 93.1%. The LLQ of BNP7787 in urine was 0.8 μ*M* and of mesna 1.6 μ*M*. The within- and between-day accuracy and precision of the assay for BNP7787 and mesna was lower than 15%. The stability of mesna in urine increased with an increasing concentration of mesna, lower temperature and addition of EDTA (1 g/l) and hydrochloric acid (0.2 *M*). BNP7787 in urine was stable for at least 24 h at temperatures in the range of −20°C up to 37°C and independent of the concentration. The developed assays are currently applied for samples of patients with solid tumors participating in a phase I trial of BNP7787 in combination with cisplatin.

Keywords: BNP7787; Dimesna; Mensa

1. Introduction

Cisplatin is an important and frequently used drug for the treatment of solid tumors. The disulfide BNP7787 (dimesna) is believed to be a non-toxic novel protector against cisplatin-induced toxicities, including nausea, vomiting, dose-limiting nephrotoxicity, and neurotoxicity, without reducing antitumor activity [1]. This selective protection is probably caused by mesna, the only metabolite of BNP7787, which is selectively formed in the kidneys, intestines and probably in the bone marrow by

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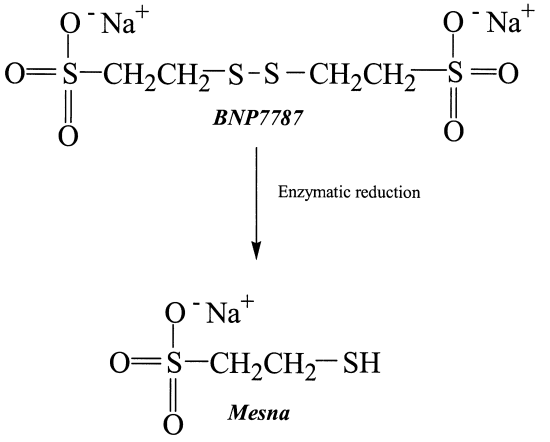

Fig. 1. Reduction of BNP7787 to mesna by the kidneys, intestine, liver and probably bone marrow.

enzymatic reduction [2] (Fig. 1). The high concentration of mesna in the kidneys can locally inactivate cisplatin by forming non-toxic platinum complexes and thereby preventing cisplatin-induced nephrotoxicity without interfering with the antitumor activity of cisplatin.

BNP7787 has recently entered a phase I clinical trial in cancer patients. To investigate the pharmacokinetics and metabolism of this new protector, a selective, sensitive and accurate analysis is required to determine the concentration of BNP7787 and mesna in plasma and urine of these patients. Up to the present a number of techniques have been used. However, the colorimetric assay of Ellman [3] is not very selective, because it also determines the endogenous thiols present in plasma. Ultraviolet detection of mesna [4] in biological fluids is not sensitive enough to determine this compound in plasma. Sidau and Shaw [5] have used a post-column derivatization, whereas Gatti et al. [6] have used a more sensitive fluorogenic pre-column derivatization. These methods have only been applied to urine samples [5] and pharmaceutical formulations [6]. Moreover, pre- or post-column derivatization is not always readily available. Up to now, high-performance liquid chromatography (HPLC) with electrochemical detection [7,8] has been preferred [5], because mesna can be detected selectively by oxidation of the thiol group. BNP7787 (dimesna) can be determined indirectly by reducing the disulfide to mesna first. This report describes a simple, accurate and sensitive method to determine mesna and BNP7787 after reduction with sodium borohydride in plasma and urine. The sample is injected into a HPLC system after a simple deproteinization (plasma) or dilution (urine) step. A sensitive electrochemical detector provided with a wall-jet gold electrode is used for the detection of mesna. The method is applied to determine the stability of (di)mesna in urine and deproteinized plasma and to analyze (di)mesna in plasma and urine of patients.

2. Materials and methods

2.1. Chemicals

BNP7787 (dimesna, disodium-bis-2-mercaptoethanesulfonate) was provided by BioNumerik Pharmaceuticals (San Antonio, TX, USA). Mesna (sodium 2-mercaptoethanesulfonate) was obtained from Sigma (St. Louis, MO, USA), acetic acid, cysteaminechloride, *o*-phosphoric acid (85%), disodium hydrogenphosphate dihydrate and sulfuric acid were from Merck (Darmstadt, Germany). EDTA disodium salt, sodium borohydride and sodium hexametaphosphate were from J.T. Baker (Deventer, The Netherlands). Tetrabutylammonium dihydrogenphosphate (1.0 *M* in water) was from Aldrich (Milwaukee, WI, USA). Trisodium citrate dihydrate and sodium hydroxide were obtained from Riedel-de Haën (Seelze, Germany). Water from a Millipore Milli-Q system (Etten-Leur, The Netherlands) was used throughout this work.

2.2. Chromatography

The HPLC system consisted of a Promis II autosampler with a cooled tray (4°C) (Spark Holland, Emmen, The Netherlands), a 400 solvent delivery system, a degasser Model GT-103 (both from Separations Analytical Instruments, H.I. Ambacht, The Netherlands) and a Decade wall jet electrochemical detector with a gold working electrode set at an operating potential of +1.00 V relative to an Ag/AgCl reference electrode (Antec Leyden, Leiden, The Netherlands). For the acquisition and processing of the data a Gyncotek

Chromeleon Chromatography Data System (Gyncotek, Softron, Germering, Germany) was used.

A 40-μl volume of the standards, quality controls (QCs) and samples was injected onto a Phenomenex Customsil 5 ODS-4 column (100×4.6 mm; Bester, Amstelveen, The Netherlands) preceded by a guard column with pellicular C_{18} refill (Alltech, Deerfield, IL, USA). The mobile phase was an aqueous solution of trisodium citrate dihydrate (0.1 *M*), tetrabutylammonium dihydrogenphosphate (1.0 m*M*) and cysteamine (0.1 μ*M*), adjusted to pH 3.5 with 85% *o*-phosphoric acid. Adding a small amount of cysteamine to the mobile phase could prevent a slight increase in the mesna signal during the first injections, which was most probably caused by adsorption of mesna to the column material.

The flow-rate used was 1 ml/min and the column was kept at a temperature of 36°C. The analysis time of one sample was 12.5 min.

2.3. Matrices

2.3.1. Plasma

EDTA plasma was prepared from blood of healthy volunteers collected in EDTA containing tubes (K3 EDTA; Becton Dickinson Vacutainer Systems, Plymouth, UK).

To obtain deproteinized human plasma, one volume of EDTA-plasma was added to one volume of 0.33 *M* sulfuric acid and one volume of sodium hexametaphosphate (5 g/100 ml) and mixed well. After centrifugation for 2 min at 12 000 *g*, the supernatant was transferred to polypropylene micro test tubes and stored at −20°C until use. The deproteinized plasma was used for the preparation of the calibration samples and QC samples.

2.3.2. Urine

Urine from healthy volunteers was used for preparing calibration samples and QC samples.

2.4. Calibration samples and quality control samples

The calibration samples and QC samples were prepared in duplicate on the day of use in blank deproteinized plasma or urine. It was not possible to prepare the QC samples in plasma or urine in advance, because of the fast formation of dimesna after addition of mesna and because of the binding of mesna to endogenous thiols and proteins.

2.4.1. Calibration and quality control samples

For the analysis of mesna in plasma, calibration samples of 0.63, 1.25, 3.2, 10, 32, 100 and 320 μ*M* were prepared in deproteinized plasma from a mesna stock solution of 12.18 m*M*. This and all other stock solutions were prepared in an aqueous solution of EDTA of 1 g/l. All stock solutions were stored at 4°C and were stable for at least 2 months at 4°C [7]. Mesna QC samples of 2, 60 and 240 μ*M* were prepared by diluting stock solutions of 40 μ*M*, 1.2 m*M* and 4.8 m*M* 20 times with deproteinized plasma on the day of use. Before injection, 750 μl of the samples was added to 150 μl of citrate–NaOH buffer (mixture consisting of 1 ml 5.0 *M* sodium hydroxide and 4 ml 0.5 *M* trisodium citrate buffer, pH 3.0) to increase the pH of the samples in order to prevent fast deterioration of the analytical column.

BNP7787 calibration samples of 1.6, 5, 16, 50, 160, 320 and 500 μ*M* were freshly prepared in deproteinized plasma from a BNP7787 stock solution of 6.13 m*M*. QC samples of BNP7787 of 2, 60 and 400 μ*M* were prepared by diluting stock solutions of 40 μ*M*, 1.2 m*M* and 8.0 m*M* 20 times with deproteinized plasma on the day of use. The samples were then subjected to reduction as described below.

Calibration samples of mesna in urine were prepared by spiking blank urine with appropriate amounts of a mesna stock solution of 304.5 m*M* followed by a 50 times dilution with mobile phase. The calibration samples obtained were 0.63, 1.25, 3.2, 10.0, 32.0, 100 and 250 μ*M* mesna in diluted urine. QC samples of mesna of 1, 25 and 240 μ*M* were freshly prepared by diluting stock solutions of 1, 25 and 240 m*M* 20 times with urine and thereafter 50 times with mobile phase. The samples were ready to be injected onto the HPLC system.

Calibration samples of BNP7787 in urine were prepared freshly by spiking blank urine with appropriate amounts of a 240 m*M* BNP7787 stock solution followed by diluting the urine 50 times with mobile phase. The concentrations obtained were 0.8, 1.6, 5.0, 16.0, 50.0, 160, 500 and 1200 μ*M* dimesna in diluted urine samples. QC samples of BNP7787 of 1.25, 32 and 800 μ*M* were prepared freshly before

each run by diluting stock solutions of 1.25, 32 and 800 m*M* 20 times with urine and thereafter 50 times with mobile phase. The samples were then subjected to reduction as described below.

2.4.2. Reduction of BNP7787 (dimesna) to mesna

BNP7787 was quantified as mesna after reduction with sodium borohydride. For that purpose 100 μl deproteinized plasma or urine diluted with mobile phase was added to 100 μl 1% EDTA (dissolved in a mixture of 5.0 ml 0.5 *M* disodium hydrogenphosphate and 2.2 ml 1.0 *M* sodium hydroxide) and 100 μl 1.06 *M* sodium borohydride solution. This mixture was vortex-mixed thoroughly and incubated for 30 min at 50°C. After cooling to room temperature, 200 μl 1.74 *M* acetic acid was carefully added to the mixture. The sample was then ready to be injected onto the HPLC system. The amount of BNP7787 was calculated by $([\text{mesna}]_{\text{after reduction}} - [\text{mesna}]_{\text{before reduction}})/2$.

2.5. Calculations

Each calibration sample was analyzed in duplicate. The mean value of the peak heights at each calibration level was used to calculate a linear calibration line. A weighting factor of $1/x$ was used for the mesna assay in plasma and urine and also for the assay of BNP7787 in plasma. For the assay of BNP7787 in urine a weighting factor of $1/x^2$ was used. The concentrations of the QC and patient samples were calculated from the measured peak heights and the calculated regression parameters of the calibration line.

2.6. Assay validation

The assays of mesna and BNP7787 (dimesna) in plasma and urine were validated by measuring the recovery, lower limit of quantitation (LLQ), linearity and within- and between-day accuracy and precision.

2.6.1. Recovery of mesna and BNP7787 in plasma and urine

The recoveries of mesna and BNP7787 in plasma and urine were determined by spiking blank EDTA plasma or urine. Immediately after spiking, the samples were deproteinized or diluted with mobile phase as described before. The recoveries were determined by a sixfold analysis of QC samples in plasma with concentrations of 6, 180 and 1200 μ*M* BNP7787 and 6, 180 and 720 μ*M* mesna or QC samples in urine with concentrations of 62.5 μ*M*, 1.6 and 40 m*M* BNP7787 and 50 μ*M*, 1.25 and 12 m*M* mesna and samples with the same concentration in an aqueous EDTA solution instead of plasma or urine. The recovery was calculated for each concentration level as the percentage of the peak heights obtained for the plasma or urine samples compared to that obtained for an equal amount of mesna or BNP7787 in the aqueous EDTA solutions.

2.6.2. Lower limit of quantitation

The LLQ was the lowest concentration of mesna or BNP7787 that could be measured with acceptable accuracy and precision (<20%). The calibration samples used for the determination of the LLQ were analyzed in triplicate, with extra calibration samples in the lowest range.

2.6.3. Linearity

The linearity of a calibration line was determined by calculating the correlation coefficient of the line and the residuals of the calibration samples. A calibration line was considered linear when the correlation coefficient (r^2) was higher than 0.99 and when the signs of the residuals were randomly distributed over the whole concentration range. The analytical outcome of a series was accepted when the accuracy and precision of the calibrators and QC samples were less than 15% with exception of the LLQ for which an error of maximal 20% was excepted [9]. Student's *t*-test was applied to the following linear model [9,10] to test if the assay was subjected to translational or rotational bias:

$$\text{measured concentration} = \alpha + \beta \text{nominal concentration} + \epsilon \quad (1)$$

in which the intercept, α, represents the translational bias, the slope, β, the rotational bias and ϵ the random measurement error. The intercept and slope were estimated by regression (*a* and *b* values, respectively). To test if the likely range of a includes zero and/or the range of *b* includes 1 a *t*-test of each estimate was performed:

$$t_a = a/\text{S.E.}(a) \text{ and } t_b = (b-1)/\text{S.E.}(b) \quad (2)$$

in which S.E.(a) and S.E.(b) are the standard errors of a and b, respectively. No significant bias is detected when the values of t_a and t_b are less than the tabulated critical values.

Statistical Product and Service Solutions (SPSS) for Windows, version 9.0.1 (SPSS, Chicago, IL, USA) was used to perform all statistical calculations.

2.6.4. Within- and between-day accuracy and precision

The accuracy was calculated as the mean observed concentration expressed as percentage of the nominal concentration. The precision was calculated as the standard deviation of the observed concentrations, expressed as percentage of the mean observed concentration. The within-day analysis was performed with the QC samples in sixfold on 1 day, whereas the between-day analysis was done in duplicate on at least 6 different days.

To detect and correct a possible gradual change in detection signal during a run, the calibration standards (C), QC and patient (S) samples were analyzed in duplicate in the following order (''in mirror image''): $C_1, \ldots C_n$, $QC_1, \ldots QC_3$, $S_1, \ldots S_n | S_n, \ldots S_1$, $QC_3, \ldots QC_1$, $C_n, \ldots C_1$.

2.7. Stability of mesna and BNP7787 in deproteinized plasma and urine

The stability of mesna and BNP7787 in deproteinized EDTA plasma (stability samples of 2 and 240 μ*M* mesna and 2 and 400 μ*M* BNP7787) and in urine (stability samples of 50 μ*M* and 12 m*M* mesna and 62.5 μ*M* and 40 m*M* BNP7787) was determined during storage at −20°C. The concentrations of the stability samples were the same as the lowest and highest QC samples used in the assays. The stability samples were stored in aliquots of 1 ml at −20°C. Samples were analyzed in duplicate as described above. Mesna and BNP7787 deproteinized plasma samples were analyzed at various time points up to 35 days after storage. The mesna urine samples were analyzed up to 29 days, whereas the BNP7787 urine samples were analyzed over a period of 122 days.

The stability of mesna and BNP7787 in urine was also determined at different temperatures. The stability samples of 50 μ*M* and 12 m*M* mesna in urine were diluted 50 times with mobile phase after standing at 4°C, room temperature or 37°C for 0, 1, 2, 4, 6, 24 and 48 h. The stability samples of BNP7787 (62.5 μ*M* and 40 m*M*) in urine were analyzed at 0, 1, 2, 4, 6, 24 and 96 h after the start of incubation. The stability of 50 μ*M* mesna was also determined in urine containing EDTA (1 g/l) or HCl (0.2 *M*) and in urine containing EDTA (1 g/l) + HCl (0.2 *M*) during incubation at 37°C and during storage at −20°C. During incubation at 37°C the stability samples were analyzed at the same time points as mesna in urine without addition of EDTA and/or HCl. After storage at −20°C the mesna samples were analyzed over a period of 21 days.

The stability samples were analyzed at the indicated times immediately after injection of a reference sample with the same mesna or BNP7787 concentration to be independent of possible changes in detection signal during the experiments. In the reference samples plasma or urine was replaced by an aqueous EDTA (1 g/l) solution. The reference samples, which are stable for at least 2 months at 4°C [7], were stored at 4°C during all stability experiments. The stability was expressed as the stability ratio, i.e., the percentage of the peak height obtained for the stability sample compared to the peak height obtained for the reference sample both with an equal amount of mesna or BNP7787. The stability sample was considered stable during a certain period of time when the ratio differed less than 15% from the ratio at the start.

The mesna stability samples were also analyzed after reduction with sodium borohydride, to investigate whether disulfide (i.e., BNP7787 or a mixed disulfide like mesna-cysteine) formation takes place. For these measurements samples with the same amount of mesna in aqueous EDTA were used as a reference. The BNP7787 stability samples were also measured without reduction with sodium borohydride, to verify a possible production of mesna. For this purpose, a reference sample of 2 μ*M* mesna was used for the BNP7787 deproteinized plasma stability samples and a 50 μ*M* mesna reference sample for the BNP7787 urine stability samples. All stability experiments were performed in duplicate.

For the stability samples, a semilogarithmic plot was made of the calculated stability ratios vs. time.

The plots were fitted with the least-squares method. If the plot was considered linear then the first-order rate law was obeyed for loss of (total) mesna:

$$-\mathrm{d[ratio\ mesna]}/\mathrm{d}t = k_{\mathrm{obs}} \cdot \mathrm{[ratio\ mesna]} \quad (3)$$

in which k_{obs} represents the observed first-order rate constant for loss of mesna.

Integration of Eq. (3) yields:

$$\ln\,\mathrm{[ratio\ mesna]}_t = \ln\,\mathrm{[ratio\ mesna]}_{t=0} - k_{\mathrm{obs}}t \quad (4)$$

leading to the half-life of mesna:

$$t_{1/2,\,\mathrm{obs}} = \ln 2/k_{\mathrm{obs}} \quad (5)$$

2.8. Patient samples

The utility of the assays is shown by the analysis of plasma and urine samples from a patient with a solid tumor who received 41 $\mathrm{g/m^2}$ BNP7787 as a 15-min intravenous infusion. Blood samples (3 ml) were collected in cooled EDTA-containing glass tubes (K3 EDTA; Becton Dickinson Vacutainer Sytems) and transported on ice. Blood samples were centrifuged at 4°C for 15 min at 3000 *g* and deproteinized immediately. The deproteinized plasma samples were stored at −20°C until analysis. Urine was collected during the first 25 h after the BNP7787 infusion in 2.5-l polyethylene bottles (Omnilabo International, Breda, The Netherlands) containing 200 ml of 2.5 *M* HCl+2.5 g sodium EDTA [11] and kept in the refrigerator (4°C) during the collection period. After measuring the volume, a portion of the urine was stored at −20°C until analysis.

3. Results and discussion

3.1. Assay validation

A representative chromatogram of blank deproteinized plasma and deproteinized plasma spiked with 100 μ*M* mesna is shown in Fig. 2. No interfering peaks were observed at the retention time of mesna. Fig. 3 shows a chromatogram of BNP7787 in deproteinized plasma after reduction to mesna with sodium borohydride. Under these conditions no interfering peaks were observed at the retention time

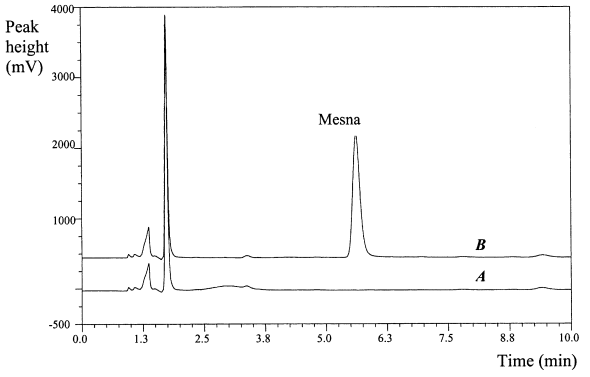

Fig. 2. A representative chromatogram of blank deproteinized plasma (A) spiked with 100 μ*M* mesna (B).

of mesna. Also in urine, no interfering peaks were detectable at the retention time of mesna before or after treatment with sodium borohydride.

The recoveries of mesna and BNP7787 in plasma and urine were independent of the concentration as shown in Table 1. In plasma, the mean recovery of mesna was 94.6% and almost complete over the whole concentration range. The mean recovery of BNP7787 from the plasma samples was 100.6%. In urine, the mean recoveries of mesna and BNP7787 were 93.1% and 94.1%, respectively.

Hydrodynamic voltamograms showed a maximum response for mesna at a potential of +1.00 V vs. Ag/AgCl. The LLQs for mesna and BNP7787 in deproteinized plasma were 0.63 μ*M* and 1.6 μ*M*, respectively. In plasma, the LLQ of mesna (1.89 μ*M*) was three- up to sixfold lower than the LLQ

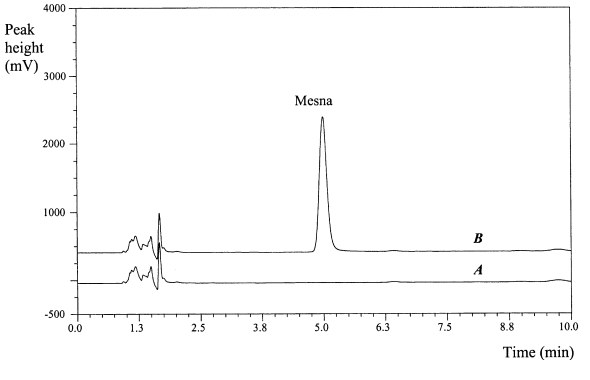

Fig. 3. A representative chromatogram of blank deproteinized plasma (A) spiked with 160 μ*M* BNP7787 measured after reduction with sodium borohydride (B).

Table 1
Recoveries of mesna and BNP7787 in plasma and urine[a]

Analyte	Plasma		Urine	
	Concentration (μM)	Recovery±SD (%)	Concentration (μM)	Recovery±SD (%)
Mesna	6	95.7±2.5	50	89.9±7.5
	180	90.5±11.8	1250	93.8±8.4
	720	97.6±2.2	12 000	95.5±4.8
BNP7787	6	105.7±2.8	62.5	94.5±3.8
	180	97.8±6.5	1600	91.1±4.4
	1200	98.4±12.8	40 000	96.7±0.7

[a] The recovery (±SD) was calculated as percentage of the peak heights obtained for the plasma or urine samples ($n=6$) compared to the mean peak height obtained for an equal amount of mesna or BNP7787 in aqueous EDTA solution ($n=6$).

reported in assays which also used HPLC and electrochemical detection to determine mesna [7,8]. A possible explanation for this difference in sensitivity may be that our procedure used an electrochemical detector provided with a gold working electrode using a wall jet configuration instead of a glassy carbon electrode with a flow-through design. The LLQ of BNP7787 (4.8 μM) in plasma was comparable to the LLQ with the method of El-Yazigi et al. [8]. The accuracy and precision of the mesna and BNP7787 calibration samples were within 15% of the nominal values with exception of the LLQ. The linearity of the assay ranged from 0.63 to 320 μM for mesna and from 1.6 to 500 μM for BNP7787. For both assays the correlation coefficient of the calibration line was better than 0.999. No significant translational and rotational bias could be detected, because the t_a and t_b values of all mesna and BNP7787 calibration curves in deproteinized plasma were less than the tabulated critical values, indicating that $a \approx 0$ and $b \approx 1$.

Our procedure to determine dimesna in plasma differs from reported methods [7,8]. In our assay, dimesna (BNP7787) was determined in plasma after a deproteinization step followed by reduction with sodium borohydride to mesna, i.e., mesna covalently bound to proteins was removed before reduction. Hence, the determined dimesna concentration, which was calculated by $([\text{mesna}]_{\text{after reduction}} - [\text{mesna}]_{\text{before reduction}})/2$, is a right estimate of the concentration of dimesna if the mixed mesna disulfides (e.g., mesna-glutathione and/or mesna-cysteine), which are also reduced by sodium borohydride, are present in a negligible concentration compared to the dimesna concentration. Earlier reported methods [7,8], however, also reduces mesna covalently bound to proteins, because they determined dimesna in plasma without deproteinization. Therefore these methods will find a higher dimesna concentration compared to our method, because approximately 10% of administered mesna is covalently and/or electrostatically bound to plasma proteins [12–14].

The LLQs of mesna and BNP7787 in urine diluted 50-times with mobile phase were 0.63 and 0.8 μM, respectively. The accuracy and precision of both assays were $<20\%$. The linearity ranged from 0.63 to 250 μM for mesna and from 0.8 to 1200 μM for BNP7787. No significant translational and rotational bias could be detected, because the t_a and t_b values of all mesna and BNP7787 calibration curves in urine were less than the tabulated critical values.

Our procedure to correct for a possible gradual decrease in detector signal during a run differs from an earlier reported method [7], in which each sample was measured between two injections of mesna in water to correct for changes in detector sensitivity. This method requires a lot of extra samples that have to be injected and therefore causes a large increase in analysis time. Furthermore, the stability of the reference injection of mesna in water has to be questioned, because mesna can readily be oxidized to dimesna under the circumstances used. In our assay, however, we measured the duplicates of all samples in ''mirror-image'' (see Materials and methods). With this ''mirror-image'' method we were able to detect

and correct for a possible gradual decrease in detection signal without the need for additional steps such as extra reference samples [7].

The within- and between-day accuracies and precisions for the mesna and BNP7787 assay in deproteinized plasma are summarized in Table 2. The within-day accuracies for the mesna and BNP7787 assay were within 12 and 2% of the nominal values, respectively. The between-day accuracy was within 9% of the nominal values for mesna and within 4% of the nominal values for BNP7787. The precision for the within-day analysis of mesna was better than 5% and of BNP7787 better than 3%. Between-day precisions of the mesna and BNP7787 assay were better than 5 and 6%, respectively. Both accuracy and precision were well within the criteria [9]. The within-day accuracy and precision for the mesna assay in urine were within 12 and 11%, respectively; for the BNP7787 assay they were within 15.2% and 2%, respectively. For both assays in urine the between-day accuracy and precision were better than 11%.

3.2. Stability of mesna and BNP7787

The stability of mesna and BNP7787 (dimesna) in urine and deproteinized plasma is important for storage conditions and the fate of the drugs during their stay in the bladder of the patients. Several clinical studies in patients and volunteers show different ways of collecting urine after mesna [5,15–21] or dimesna administration [22] without mentioning the stability of the samples under the circumstances used. The stability of mesna in plasma has already been investigated [7] except for the stability of mesna and dimesna in deproteinized plasma during storage at −20°C. Therefore, we investigated the stability of mesna and BNP7787 in urine under various conditions and in deproteinized plasma at −20°C only.

Because semilogarithmic plots of the calculated stability ratios vs. time were linear, first-order rate constants (k_{obs}) could be calculated for loss of mesna according to Eq. (3). The k_{obs} values and the derived half-lives ($t_{1/2,\ obs}$) (Eq. (5)) of mesna in urine are summarized in Table 3. The half-life of mesna increased with decreasing temperature and increasing concentration of mesna. The half-lives measured in the mesna stability samples after reduction with sodium borohydride (i.e., total mesna) were at least 1.5-fold higher than the half-lives before reduction. Therefore the loss of mesna could largely be explained by the formation of disulfides (i.e., BNP7787 or a mixed disulfides like mesna-cysteine). Yet a small decrease of the total mesna concentration was seen especially at 37°C, i.e., a lowest half-life for total mesna of 2.6 days.

The poor stability of 50 μ*M* mesna in urine at 37°C indicates that it is very important that patients void their bladder soon and often after administration of BNP7787 to reduce the residence time of mesna in the bladder. The stability of 50 μ*M* mesna at room temperature and 4°C is also not sufficient enough to collect and store urine at these temperatures for 25 h. Therefore the recommendation to add EDTA and hydrochloric acid in the urine to prevent oxidation of

Table 2
Within- and between-day accuracy and precision at three concentrations of mesna and BNP7787 in deproteinized plasma

Analyte	Concentration (μ*M*)	Accuracy (%)		Precision (%)	
		Within-day ($n=6$)	Between-day ($n=6$)	Within-day ($n=6$)	Between-day ($n=6$)
Mesna	2	88.9	91.4	4.1	2.7
	60	109.1	106.1	2.3	4.1
	240	104.0	100.3	0.9	4.0
BNP7787	2	98.5	103.5	2.9	5.4
	60	100.7	100.5	1.6	2.4
	400	100.4	97.4	2.4	4.7

Table 3
First-order rate constants (k_{obs}) and half-life times ($t_{1/2, obs}$) of mesna in urine determined for two concentrations (50 μ*M* and 12 m*M*), at different temperatures (37°C, room temperature, 4°C and −20°C) and with addition of EDTA (1 g/l), HCl (0.2 *M*) or EDTA (1 g/l)+HCl (0.2 *M*)

Addition	Mesna concentration	Temperature (°C)	k_{obs} (day^{-1})	$t_{1/2, obs}$ (day)
None	12 m*M*	−20	0.004	170.3
		4	0.028	24.7
		Room temperature	0.078	8.9
		37	0.147	4.7
	50 μ*M*	−20	0.033	21.3
		4	0.147	4.7
		Room temperature	0.497	1.4
		37	1.494	0.5
EDTA	50 μ*M*	−20	0.045	15.4
		37	1.163	0.6
HCl	50 μ*M*	−20	0.017	39.9
		37	0.240	2.9
EDTA+HCl	50 μ*M*	−20	0.007	95.9
		37	0.155	4.5

mesna during collection was investigated [11]. Addition of EDTA (1 g/l) to urine did not increase the half-life of mesna at different temperatures, whereas addition of 0.2 *M* HCl to urine increased the half-life of mesna at 37°C approximately six times (Table 3). The highest stability of 50 μ*M* mesna in urine was obtained by adding EDTA (1 g/l)+HCl (0.2 *M*), i.e., the half-life of mesna was 4.5 days at 37°C. The total mesna concentration remained unchanged during the 48 h measured.

The stability of BNP7787 in urine was independent of the concentration used. At 37°C the half-life of BNP7787 was higher than 18 days, whereas the half-life of BNP7787 during storage at −20°C was higher than 500 days. Less than 2% of mesna could be detected at the highest temperature (37°C) in the BNP7787 urine stability samples.

Two and 240 μ*M* mesna in deproteinized plasma and stored at −20°C had a half-life of 46 days and 110 days, respectively. Under these conditions 2 and 400 μ*M* BNP7787 were stable for at least 35 days. No free mesna could be detected in the BNP7787 stability samples. In plasma, however, mesna had a very short half-life [7]. It is therefore highly recommended to deproteinize blood samples quickly after collection from the patient.

3.3. Patient samples

The assays of mesna and BNP7787 in plasma and urine were applied to samples of a patient collected at various time points after receiving the highest dose of BNP7787 in the phase I trail, i.e., 41 g/m^2 as a 15 min intravenous infusion. At the end of the BNP7787 infusion the concentration of BNP7787 in plasma was 14.7 m*M* and the concentration of mesna 34.4 μ*M*. The highest plasma concentration of mesna (i.e., 282.5 μ*M*) was reached 2.25 h after the start of the BNP7787 infusion. These mesna concentrations were within the dynamic range of the mesna assay. The concentrations of BNP7787 were above the dynamic range for about 5 h after the end of the infusion. The dynamic range of the BNP7787 assay could not be extended because the calibration line was not linear above the highest calibration sample. Therefore samples had to be analyzed after an appropriate dilution with blank deproteinized plasma. Based on the sensitivity of the assays it was possible

to measure plasma levels of mesna and BNP7787 up to 17 and 14 h after starting the BNP7787 infusion, respectively. The concentrations of free mesna and BNP7787 in the 25-h urine were 21.6 and 84.9 m*M*, respectively. These concentrations were beyond the dynamic range of the assays, indicating that mesna and BNP7787 could easily be measured in the urine for 25 h or even longer.

4. Conclusion

A sensitive, accurate and precise method has been developed to determine BNP7787 and mesna in plasma and urine of cancer patients receiving BNP7787. This method allowed the measurement of the stability of (di)mesna in plasma and urine in clinically relevant concentrations. The method will be applied further to analyze (di)mesna plasma and urine samples of patients treated with BNP7787.

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