Simultaneous Determination of BNP7787 and its Metabolite Mesna in Plasma and Tissue by Micro-HPLC with a Dual Electrochemical Detector

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ABSTRACT: Sensitive, accurate, and precise assays are described to determine BNP7787 (disodium 2,2′-dithio-bis-ethane sulfonate) and its metabolite mesna (sodium 2-mercaptoethane sulfonate) simultaneously in plasma and tissue by micro-high-performance liquid chromatography (HPLC) with dual electrochemical detection. After separation of BNP7787 and mesna by micro-HPLC, the disulfide BNP7787 was reduced to mesna by a reactor cell with a glassy carbon working electrode (−1.6 V versus Hy-REF). At the second electrode, which consisted of a gold wall-jet electrode, the mesna generated from BNP7787 and the mesna already present in the samples were detected (+0.85 V versus Ag/AgCl). The lower limit of quantification (LLQ) of both compounds was 3 μM in plasma and 20 nmol/g in tissue. The dynamic range of the assay in plasma was 3–120 μM for mesna and 15–1200 μM for BNP7787. In tissue, the dynamic range was 20–2000 nmol/g for both compounds. The recovery of mesna from plasma and tissue ranged from 61.4 to 90.5% and 82.7 to 90.2%, respectively, and seemed to be concentration dependent. The recovery of BNP7787 from plasma and tissue was complete (i.e., 101.5 and 96.4%, respectively). The within- and between-day accuracy and precision for the plasma and tissue assay were within 14 and 7%, respectively. The utility of the assay was shown by determination of the stability of mesna and BNP7787 in a kidney sample of a rat and by analysis of plasma samples obtained from a patient receiving 18.4 g/m^2 BNP7787 as a 15-min intravenous infusion. J Pharm Sci 92:1040–1050, 2003

Keywords: BNP7787; mesna; micro-HPLC; dual electrochemical detector; plasma; tissue

INTRODUCTION

Cisplatin is an important and frequently used drug in the treatment of solid tumors, including those of the lung, head and neck, ovary, and testis. The water-soluble disulfide BNP7787 (disodium 2,2′-dithio-bis-ethane sulfonate) is under investigation as a novel nontoxic agent to protect against cisplatin-induced toxicities, such as nausea, vomiting, dose-limiting nephrotoxicity, and neurotoxicity.[1] In the kidneys, intestine, and liver, BNP7787 is enzymatically converted into its active metabolite mesna (sodium 2-mercaptoethane sulfonate)[2–4] (Figure 1). In turn, the thiol mesna can locally inactivate cisplatin and its hydrated complexes by forming nontoxic platinum complexes and thereby preventing cisplatin-induced nephrotoxicity without reducing antitumor activity of cisplatin.

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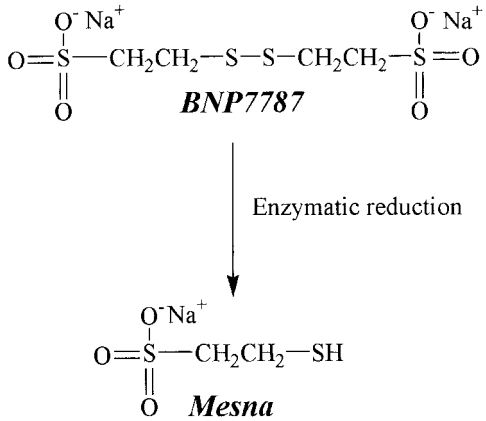

Figure 1. Enzymatic reduction of BNP7787 into mesna in the kidney, intestine, and liver.

Up to now, mesna is determined in biological fluids by high-performance liquid chromatography (HPLC) with electrochemical detection.[5–9] BNP7787 (the symmetrical disulfide of mesna) is measured indirectly as mesna after precolumn reduction with sodium borohydride. With this procedure, mixed disulfides with mesna (e.g., mesna-cysteine, mesna-glutathione) will also contribute to the total amount of mesna generated by sodium borohydride. Therefore, there is a need for a specific assay to determine BNP7787 in plasma and tissue.

Kleinman and Richie[10] developed an HPLC method using dual electrochemical detection with mercury-based electrodes, which were difficult to prepare and maintain. Recently, we described HPLC procedures for the determination of the thiol mesna in plasma, urine, and tissue[8,9] using a sensitive and easy to maintain wall-jet gold electrode. Therefore, our objective was to use this type of electrode in a dual electrochemical detection system for developing and validating a procedure for the simultaneous determination of BNP7787 and mesna in plasma and tissue after separation by micro-HPLC. The utility of this assay is shown by the analysis of plasma samples obtained from a patient receiving 18.4 g/m^2 BNP7787 as a 15-min intravenous (iv) infusion and by the determination of the stability of mesna and BNP7787 in a kidney sample of a rat during storage under various conditions.

EXPERIMENTAL SECTION

Chemicals

BNP7787 (disodium 2,2′-dithio-bis-ethane sulfonate) was provided by BioNumerik Pharmaceuticals (San Antonio, TX) and mesna (sodium 2-mercaptoethane sulfonate) was obtained from Sigma Chemicals (St. Louis, MO). EDTA disodium salt and sodium hexametaphosphate were from J.T. Baker (Deventer, The Netherlands). Cysteamine chloride, trisodium citrate dihydrate, *o*-phosphoric acid (85%), hydrochloric acid, and sulfuric acid were from Merck (Darmstadt, Germany). Tetrabutyl ammonium dihydrogen phosphate (TBAP; 1.0 M in water) was from Aldrich (Milwaukee, WI) and sodium hydroxide was obtained from Riedel-de Haën (Seelze, Germany). Deionized water from a Millipore Milli-Q system (Etten-Leur, The Netherlands) was used throughout this work.

Chromatography

The micro-HPLC system consisted of a Spark Triathlon 900 autosampler with a 20-μL loop and a cooled tray (4°C; version 1.86), a Knauer K-501 pump, and a degasser model GT-103 (all from Separations Analytical Instruments, H. I. Ambacht, The Netherlands). One microliter of the samples containing BNP7787 and mesna was injected onto a Phenomenex Prodigy C18 ODS-3, 5-μ column (100 × 1.0 mm) preceded by a guard column with Phenomenex C18, 10 μ (4 × 2.0 mm; both columns are from Bester, Amstelveen, The Netherlands). Both columns were kept at a temperature of 36°C with a Spark Mistral column thermostat (Separations Analytical Instruments, H. I. Ambacht, The Netherlands). The mobile phase for the plasma analysis consisted of an aqueous solution of trisodium citrate dihydrate (0.15 M), TBAP (0.05 mM), and cysteamine (0.1 μM), adjusted to pH 3.5 with 85% *o*-phosphoric acid. For the tissue analysis, the mobile phase was almost the same except the TBAP concentration was 0.1 mM instead of 0.05 mM. The flow-rate was set at 0.05 mL/min. After separation of BNP7787 and mesna on the column, both compounds were detected with a dual amperometric electrochemical detector (dual ECD; Decade with Twin option set at 36°C; Antec Leyden, Zoeterwoude, The Netherlands). This detector consisted of a reactor cell with a glassy carbon working electrode set at an operating reduction potential of −1.60 V relative to an Hy-REF reference electrode and a wall-jet flow cell with a gold working electrode set at an operating oxidation potential of +0.85 V relative to an Ag/AgCl reference electrode in saturated KCl. At the first electrode, BNP7787 was reduced to mesna, which was subsequently oxidized at the second electrode. Mesna already

present in the sample and eluting at its own retention time was directly detected at the second electrode. For the acquisition and processing of the data, a Dionex Chromeleon Chromatography Data System (version 4.30; Dionex Softron, Germering, Germany) was used. All samples were measured in duplicate, starting with the first measurement of the calibration, quality control (QC), and patient samples, followed by the second measurement of these samples in reverse order. The samples were measured in this order to detect and automatically correct the mean of the duplicate values for a possible gradual change in signal.

Matrices

Plasma

Drug-free human EDTA-plasma was deproteinized by adding 1 volume of 0.33 M sulfuric acid and 1 volume of 5% sodium hexametaphosphate to 1 volume of EDTA-plasma. After mixing well and centrifugation (2 min at 4°C and 9000*g*), the supernatant was stored at −20°C until analysis.

Tissue

Fresh drug-free pig kidney was pulverized with a Braun Micro-dismembrator (Salm & Kipp, Breukelen, The Netherlands). An appropriate volume of aqueous EDTA (1 g/L) was added to obtain a solution of 100 mg/mL (wet weight). After 10 min of sonicating and 10 min of vortexing, the blank tissue homogenate was stored at −80°C until use.

Calibration and Quality Control Samples in Plasma

Before each run, BNP7787 and mesna calibration samples were prepared freshly in duplicate in deproteinized EDTA-plasma by adding appropriate amounts of stock solutions containing 10 mM BNP7787 and 1.0 mM mesna in aqueous EDTA (1 g/L). This procedure resulted in deproteinized plasma calibration samples in the range 5–400 μM for BNP7787 and 1–40 μM for mesna. The QC samples of 15, 75, and 300 μM BNP7787 and of 1.5, 7.5, and 30 μM mesna were prepared in duplicate by diluting BNP7787 and mesna stock solutions in aqueous EDTA (1 g/L) 20 times with deproteinized plasma on the day of use. Of each calibration and QC sample, 200 μL was added to 40 μL of a citrate/NaOH buffer (mixture 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 to ∼3.5 to prevent fast deterioration of the analytical column. Thus, the samples were ready to be injected onto the micro-HPLC system.

Calibration and Quality Control Samples in Tissue

For the analysis in tissue, the calibration samples of BNP7787 and mesna both were in the range 20–2000 nmol/g and both were prepared freshly on ice and in duplicate by spiking tissue homogenate with appropriate amounts of stock solutions containing 20 mM BNP7787 and 20 mM mesna in aqueous EDTA (1 g/L). The BNP7787 and mesna QC samples of 50, 250, and 1250 nmol/g were prepared in the same way using BNP7787 and mesna stock solutions in aqueous EDTA (1 g/L) of the same concentration but prepared at another occasion. After preparation, 1 volume of calibration or QC sample was immediately deproteinized by adding 1 volume of cold 0.33 M sulfuric acid and 1 volume of cold sodium hexametaphosphate (5 g/100 mL). After vortexing well and centrifugation for 2 min at 9000 rpm and 4°C, 200 μL of supernatant was added to 40 μL of citrate–NaOH buffer (mixture of 1 mL of 5.0 M sodium hydroxide and 4 mL of 0.5 M trisodium citrate buffer, pH 3.0). The resulting samples were ready to be injected onto the micro-HPLC system.

Calculations

The mean values of the peak heights at each calibration level were used to calculate the linear regression parameters for plasma using a weighting factor $1/X$. The mean values of the peak areas, without weighting factor, were used for the tissue analysis. BNP7787 and mesna concentrations were calculated by interpolation of the peak heights or peak areas of the QC, rat, and patient samples on the calibration line.

Optimization of the Assay

Hydrodynamic Voltammogram

The optimal reduction and oxidation potentials were determined by measuring hydrodynamic voltammograms. To obtain the optimal reduction potential, samples containing 200 μM BNP7787 in mobile phase (0.15 M trisodium citrate dihydrate, 0.05 mM TBAP, and 0.1 μM cysteamine, adjusted to pH 3.5 with 85% *o*-phosphoric acid) were measured at different reduction potentials from

−1.0 to −1.8 V (versus Hy-REF) and a constant oxidation potential of +1.0 V (versus Ag/AgCl). The optimal oxidation potential was determined with samples containing 100 μM mesna and 200 μM BNP7787. The peak heights and background current were measured at different oxidation potentials from +0.75 to +1.0 V. The reduction potential was kept constant at −1.6 V.

Optimization of Chromatographic Conditions

The chromatographic conditions were optimized by changing independently the concentration of trisodium citrate, which was used in the buffer; the concentration of TBAP, which was used as the ion-paring compound; and the pH of the mobile phase and the column temperature.

Assay Validation

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

Selectivity

Retention times of the endogenous thiols [glutathione (GSH), cysteine (Cys), homocysteine (HCys), and methionine], including their disulfides, were determined to check whether these compounds interfered with the determination of mesna and BNP7787.

Recovery of Mesna and BNP7787 from Plasma and Tissue

Recoveries of mesna and BNP7787 were determined from the QC samples in tissue and plasma as well as blank deproteinized EDTA-plasma spiked with mesna or BNP7787 to obtain concentrations of 50, 250, and 1250 nmol/g for mesna and BNP7787 in tissue, 4.5, 22.5, and 90 μM for mesna in plasma, and 45, 225, and 900 μM for BNP7787 in plasma. The concentrations of the mesna and BNP7787 QC samples in deproteinized plasma were three times lower than those in plasma. Samples with the same concentration in aqueous EDTA instead of deproteinized plasma, plasma, or tissue were used as a reference. After preparation, the plasma, tissue, and EDTA samples were immediately deproteinized and further treated as described before. The recovery was calculated for each concentration level as the percentage of the mean peak height or area obtained for the deproteinized plasma, plasma, or tissue samples compared with the mean peak height or area obtained for samples with the same concentration of mesna and BNP7787 in the aqueous EDTA solution.

Lower Limit of Quantification

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

Linearity

A calibration line was considered linear when the correlation coefficient (r^2) was >0.99, when the coefficient of variation of the calibration samples was <15% (with exception of the LLQ for which a maximal error of 20% was accepted), and when the signs of the residuals were randomly distributed over the whole concentration range.[11]

A Student's t-test was applied to the following linear model[11,12] to test if the assay was subjected to translational or rotational bias:

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

where the intercept α represents the translational bias, the slope β represents the rotational bias, and ε is the random measurement error. The intercept and slope were estimated by regression (a and b value, 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:

$$t_a = a/\mathrm{SE}(a) \quad \text{and} \quad t_b = (b-1)/\mathrm{SE}(b) \quad (2)$$

in which SE is the standard error of a or b. No significant bias was detected when the values of t_a and t_b were 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.

Within- and Between-Day Accuracy and Precision

The within-day accuracy and precision were determined by measuring all QC samples of mesna and BNP7787 six times in one day, whereas the analyses for the between-day accuracy and precision were performed in duplicate on six

different days. 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.

Stability of Mesna and BNP7787 in Tissue

The stability of mesna and total mesna [i.e., mesna plus mesna disulfides (BNP7787) and mixed mesna disulfides (e.g., mesna-glutathione, mesna-cysteine)] in plasma and deproteinized plasma has been described before.[6,8] Also, the stability of mesna and total mesna in tissue during sample processing, storage, and tray stability has been described.[9] Therefore, only the stability of mesna and BNP7787 in kidney tissue during various storage conditions was investigated here with the dual ECD. The mesna and total mesna concentrations were also determined by HPLC with single ECD as described previously.[9] BNP7787 was administered iv at a dose of 1000 mg/kg to a rat. After 45 min, the kidneys were removed and immediately frozen after washing in cold PBS solution to remove the blood. Tissue homogenate and deproteinized tissue homogenate of the kidneys were prepared as described before[9] and stored in aliquots at −20 and −80°C. To determine the stability under these storage conditions, mesna and BNP7787 concentrations were determined in duplicate at day 0 and at different times up to 12 weeks of storage.

Patient Samples

The assay described in this manuscript was used to analyze mesna and BNP7787 in plasma samples of a patient with a solid tumor who received 18.4 g/m^2 BNP7787 as a 15-min iv infusion. Blood samples were collected in cooled EDTA-containing glass tubes and transported on ice. They were centrifuged at 4°C for 15 min at 3000*g* and deproteinized immediately. The deproteinized plasma was stored at −80°C until analysis.

RESULTS

Assay Optimization

Hydrodynamic Voltammogram

The optimal reduction potential of the glassy carbon working electrode was determined by a hydrodynamic voltammogram of BNP7787 in mobile phase. The oxidation potential of the wall-jet flow cell with a gold working electrode was kept constant at +1 V versus an Ag/AgCl electrode. The voltammogram showed a maximum response to background current ratio for BNP7787 at a potential of −1.6 V versus a Hy-REF electrode. A gold electrode was also tested as a working electrode. However, the gold electrode proved to be less efficient than the glassy carbon electrode.

The optimal oxidation potential of the wall-jet flow cell with gold working electrode was determined by a hydrodynamic voltammogram with a constant reduction potential of −1.6 V versus Hy-REF of the reactor cell with a glassy carbon working electrode. The results showed that the optimal responses for mesna and BNP7787 were obtained at a potential of +1.0 and +0.95 V, respectively (Figure 2). The signal-to-noise ratio was the highest at +0.85 V relative to an Ag/AgCl reference electrode and therefore this potential was selected for the assay of both compounds. A wall-jet flow cell with a glassy carbon working electrode was also examined but proved to be less sensitive than a gold working electrode.

Optimization of Chromatographic Conditions

The best separation of mesna and BNP7787 was obtained by reversed-phase ion-pairing

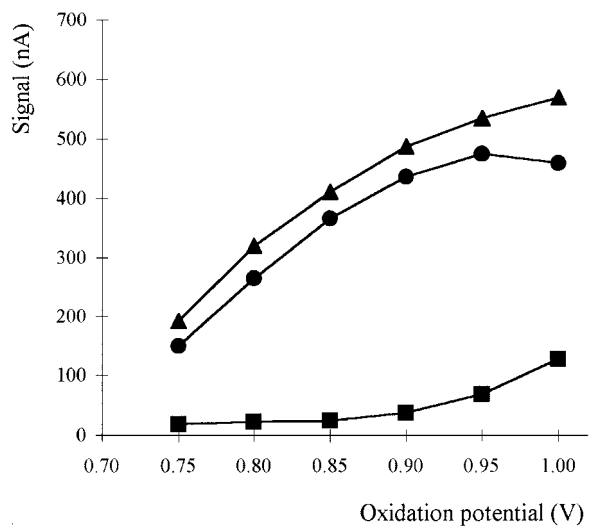

Figure 2. Hydrodynamic voltammogram of the signal response of (▲) 100 μM mesna and (●) 200 μM BNP7787 in mobile phase and (■) the background current. Conditions were as follows: reactor cell with a glassy carbon working electrode set at −1.6 V versus Hy-REF; wall-jet flow cell with a gold working electrode set at +0.75–1.00 V versus Ag/AgCl.

chromatography. Different components and parameters of the mobile phase were adjusted independently to obtain optimal separation of mesna and BNP7787 from endogenous compounds in plasma and tissue.

The mobile phase that resulted in optimal retention times of mesna and BNP7787 and separation of both compounds from endogenous compounds in plasma contained 0.15 M trisodium citrate, 0.05 mM TBAP, and 0.1 μM cysteamine. Optimal chromatographic conditions to separate mesna and BNP7787 from endogenous components in tissue were obtained with almost the same mobile phase except for a higher TBAP concentration (0.1 instead of 0.05 mM). The pH of both mobile phases was 3.5 and the temperature was kept at 36°C. Under these circumstances, chromatographic analysis of a blank deproteinized plasma sample and a deproteinized plasma sample spiked with mesna and BNP7787 showed no interfering peaks at the retention time of mesna and BNP7787 (Figure 3). Also, no interfering peaks were observed at the retention times of mesna and BNP7787 in a spiked deproteinized kidney homogenate, as shown in Figure 4.

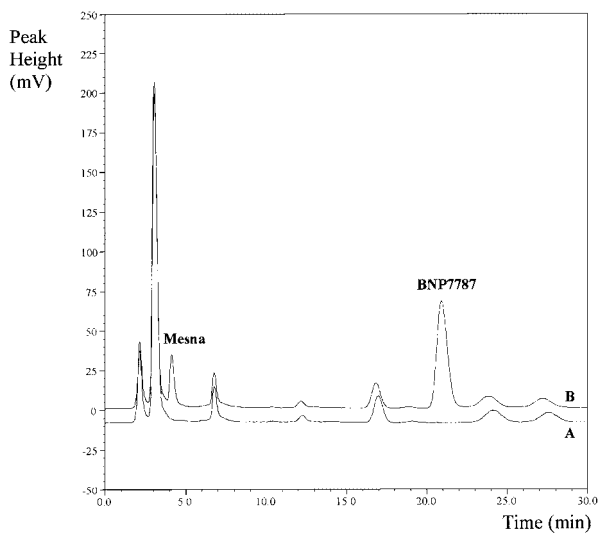

Figure 3. Chromatogram of (A) blank deproteinized plasma and (B) blank deproteinized plasma spiked with 10 μM mesna and 100 μM BNP7787.

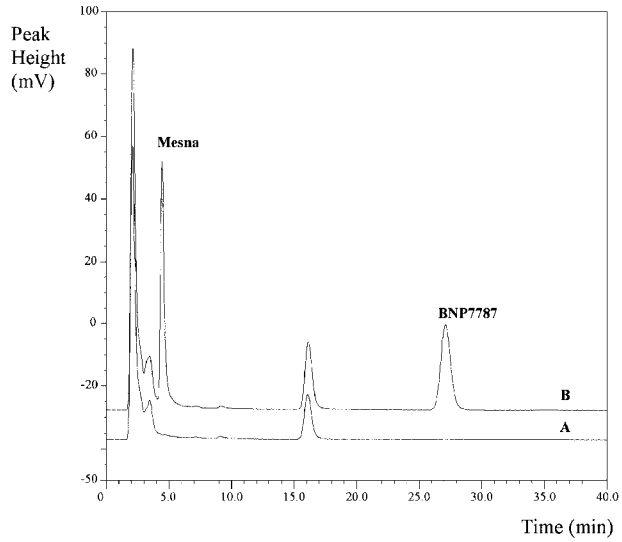

Figure 4. Chromatogram of (A) blank deproteinized kidney homogenate and (B) blank deproteinized kidney homogenate spiked with 500 nmol/g mesna and 500 nmol/g BNP7787.

Assay Validation

Selectivity

The endogenous thiols [glutathione (GSH), cysteine (Cys), homocysteine (Hcys), and methionine], like the symmetrical disulfides of GSH, Cys and Hcys did not interfere with the determination of mesna and BNP7787 in plasma and tissue. All these compounds had retention times of <3.6 min under the conditions used, whereas mesna and BNP7787 had retention times of 4.6 and 29 min, respectively.

Recovery of Mesna and BNP7787 from Plasma and Tissue

The recovery of mesna from the QC samples freshly prepared in deproteinized plasma and plasma compared to the same solutions freshly prepared in aqueous EDTA were comparable (i.e., 68.9–96.6 and 61.4–90.5%, respectively), and both recoveries seemed to be concentration dependent in the same way (Table 1). The loss due to deproteinization (i.e., the difference between the recovery of mesna from plasma and its recovery from deproteinized plasma) was minimal and independent of the concentration. The mean recoveries of BNP7787 from deproteinized plasma and from plasma were almost complete over the whole concentration range (i.e., 100.6 and 101.1%, respectively; Table 1).

From tissue, the recovery of mesna was also concentration dependent and ranged from 82.6 to 90.2%. The mean recovery of BNP7787 from tissue compared to the same concentrations in aqueous EDTA solution was 96.4% and almost complete over the whole concentration range (Table 1).

Table 1. Mean Recoveries (±SD) of Mesna and BNP7787 from Deproteinized Plasma ($n = 4$), Plasma ($n = 4$), and Tissue ($n = 6$) Compared to the Same Concentrations in an Aqueous EDTA Solution

	Deproteinized Plasma		Plasma		Tissue	
Analyte	Concentration (μM)	Recovery ± SD (%)	Concentration (μM)	Recovery ± SD (%)	Concentration (nmol/g)	Recovery ± SD (%)
Mesna	1.5	68.9 ± 9.2	4.5	61.4 ± 6.8	50	82.7 ± 4.6
	7.5	88.0 ± 6.0	22.5	78.0 ± 6.7	250	88.0 ± 1.9
	30	96.6 ± 4.3	90	90.5 ± 2.4	1250	90.2 ± 2.1
BNP7787	15	104.5 ± 11.0	45	108.4 ± 3.7	50	96.9 ± 4.1
	75	98.9 ± 6.4	225	96.7 ± 5.9	250	100.0 ± 1.6
	300	98.4 ± 7.3	900	98.2 ± 3.4	1250	92.4 ± 7.3

Lower Limit of Quantification, Linearity, and Dynamic Range

The LLQ for mesna was the same (i.e., 3 μM) as that for BNP7787 in plasma. In tissue, the LLQs for mesna and BNP7787 were also the same (i.e., 20 nmol/g).

The calibration line in deproteinized plasma was in the range 1–40 μM for mesna, 5–50 μM for BNP7787, and 50–400 μM for the higher BNP7787 concentrations. Due to dilution caused by deproteinization, the corresponding concentrations in plasma were threefold higher than measured in the deproteinized samples. The calibration lines for both mesna and BNP7787 in tissue were in the ranges 20–500 and 500–2000 nmol/g.

The accuracy and precision of the BNP7787 and mesna calibration samples were <15% over the entire concentration range and <20% at the LLQ. The correlation coefficients for the calibration lines of BNP7787 in plasma covering the high concentration range (50–400 μM) were >0.988 and for mesna and BNP7787 in the low concentration range (5–50 μM) >0.966. In tissue, the correlation coefficients of all BNP7787 and mesna calibration lines were >0.986 and >0.995, respectively. The t_a and t_b values of all calibration lines for mesna and BNP7787 (t_a and t_b were <0.03) were less than the tabulated critical values, indicating that no significant translational and rotational bias could be detected.

Within- and Between-Day Accuracy and Precision

In plasma, the within- and between-day precisions of the mesna analysis of the QC samples was >7%. The within- and between-day accuracies of these samples were within 3.3 and 2.8% of the nominal values, respectively. The within- and between-day accuracies of BNP7787 were within 6.3% of the nominal values. The within- and between-day precisions for BNP7787 ranged from 1.5–6.1 and 2.7–4.9%, respectively.

The within- and between-day accuracies and precisions of mesna and BNP7787 in tissue are summarized in Table 2. The within- and between-day accuracies of mesna and BNP7787 were within 6.4 and 13.7% of the nominal values, respectively. The within-day precisions for the QC samples for mesna and BNP7787 were >5.6 and >4.2%, respectively. The between-day precisions of mesna and BNP7787 were >3.0 and >4.7%, respectively.

Table 2. Within-Day and Between-Day Accuracy and Precision at Three Concentrations of Mesna and BNP7787 in Tissue

		Accuracy (%)		Precision (%)	
Analyte	Concentration (nmol/g)	Within-Day ($n = 6$)	Between-Day ($n = 6$)	Within-Day ($n = 6$)	Between-Day ($n = 6$)
Mesna	50	101.4	101.4	5.6	2.4
	250	105.3	104.2	4.4	3.0
	1250	106.4	104.6	3.0	2.0
BNP7787	50	113.7	103.5	4.2	4.7
	250	108.0	105.6	1.3	2.2
	1250	105.8	104.4	2.2	1.4

Stability of Mesna and BNP7787 in Tissue

Mesna in tissue homogenate containing EDTA (1 g/L) and in deproteinized tissue homogenate both stored at −80°C was stable over the 12 weeks measured. The same result was found for BNP7787. The total mesna concentration, which was measured after precolumn reduction with sodium borohydride by HPLC with single ECD,[9] was also stable. The total mesna concentration (i.e., 13.4 μmol/g) was comparable to [mesna] + 2 × [BNP7787] (i.e., 7.7 μmol/g + 2 × 2.9 μmol/g), indicating that no mixed mesna disulfides were present in the kidney of a rat 45 min after an iv bolus injection of BNP7787 at 1000 mg/kg.

The mesna concentration in tissue homogenate stored at −20°C decreased 97% in 2 weeks. The increase of the BNP7787 concentration in this homogenate corresponded with ~50% of the loss in mesna concentration. The mesna concentration in deproteinized tissue homogenate stored at −20°C showed a smaller decrease in time ($t_{1/2,obs}$ = 28 days) than the tissue homogenate itself. The increase in BNP7787 concentration could almost completely explain the loss of mesna in deproteinized tissue with time.

Mesna was also determined in the stability samples using our previously described HPLC assay with single ECD.[9] The results obtained for the mesna concentrations determined with the single ECD assay were comparable to the results from the dual ECD assay. A good correlation was found, with a slope of 0.95 and a correlation coefficient of 0.99 (Figure 5).

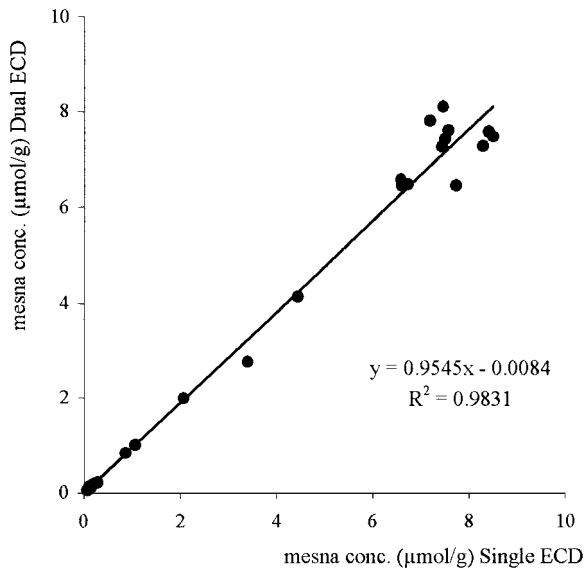

Figure 5. Comparison of the mesna concentrations measured in the stability samples either by HPLC with single ECD or HPLC with dual ECD.

Patient Samples

The developed assay was applied to determine the concentrations of mesna and BNP7787 in plasma samples of a patient receiving a 15-min iv infusion of BNP7787 at 18.4 g/m^2 (Figure 6). A maximum BNP7787 concentration of 6.2 mM was reached at the end of the infusion. A maximum mesna concentration of 191 μM was reached at ~2 h after the end of the BNP7787 infusion. Approximately 6 h after the BNP7787 infusion, the levels were decreased to 46 μM for mesna and 325 μM for BNP7787. These levels clearly indicate that our developed assay has the sensitivity and reliability to be used for pharmacokinetic purposes in patients receiving BNP7787.

DISCUSSION

Dual electrochemical detection systems have been used for the simultaneous measurement of the endogenous thiol glutathione and it disulfide, GSSG, in biological fluids.[10,13–15] The assay using amperometric electrochemical detectors provided with gold/mercury amalgam electrodes[10] was two times more sensitive for glutathione and even three times more sensitive for GSSG compared with the assays using coulometric electrochemical

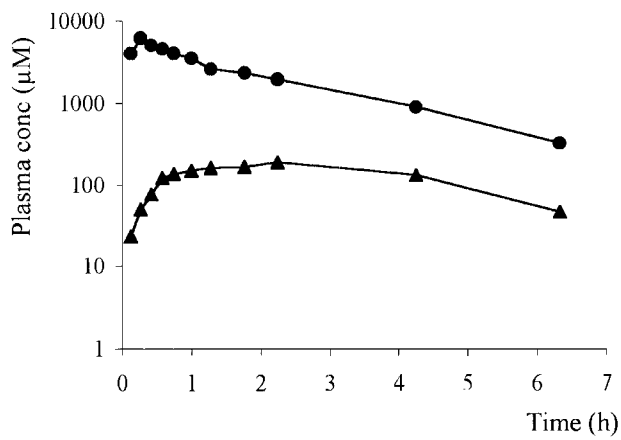

Figure 6. Semilogarithmic concentration–time curves of (▲) mesna and (●) BNP7787 in plasma of a patient during and after the administration of a 15-min iv infusion of BNP7787 at 18.4 g/m^2.

detectors provided with porous graphite electrodes.[13,14] However, the disadvantage of the gold/mercury amalgam electrodes is that they are difficult to prepare and maintain. Therefore, we investigated the use of an easy to maintain and sensitive dual amperometric electrochemical detection system to determine mesna and BNP7787 in plasma and tissue. A glassy carbon working electrode was used in the reactor cell and a sensitive gold working electrode in the wall-jet flow cell. With these cells, we obtained an LLQ of mesna in plasma of 3 μM. This value is in the range of the LLQs reported in literature for mesna (0.16–6.1 μM) when determined by HPLC with a single ECD or pre- or postcolumn derivatization followed by spectrophotometric detection.[6–8,16,17]

The LLQ of BNP7787 in plasma with our assay (i.e., 3 μM) was approximately threefold higher than the LLQ obtained by Goren et al.,[16] who determined BNP7787 by HPLC with post-column sulfitolysis. Although our LLQ is higher compared with those in the literature, our assay is sensitive enough to measure concentrations of BNP7787 in plasma samples of a patient receiving BNP7787 at 18.4 g/m^2 (Figure 6).

The LLQ of mesna in tissue (i.e., 20 nmol/g) was only twofold higher than the LLQ obtained with our previously reported assay,[9] despite the use of a 40-fold smaller injection volume due to the use of micro-HPLC. These results indicate that our new assays for mesna and BNP7787 in plasma and tissue have sensitivity that is comparable to that of the methods described before, but with the important difference that BNP7787 can be determined selectively and in the same chromatographic run as mesna. Because the injection volume was only 1 μL, only small amounts of plasma and tissue are required for the analysis of mesna and BNP7787, which allows repeated sampling of small laboratory animals and patients for pharmacokinetic purposes.

The dynamic ranges of the assay in plasma were 3–120 μM for mesna and 15–1200 μM for BNP7787. The plasma concentration–time curves of the patient who received BNP7787 at 18.4 g/m^2 (Figure 6) shows that the concentrations of BNP7787 were above the dynamic range of the assay for ~3 h after the start of the infusion, whereas the mesna concentrations were just above the dynamic range of the assay from 0.5–4.3 h after the start of the infusion. The dynamic range of the assay could not be extended because the calibration lines were not linear above the highest calibration sample. Therefore, samples had to be diluted with appropriate amounts of blank deproteinized plasma before analysis with HPLC and dual ECD. Goren et al.[16] also reported a method to determine mesna and BNP7787 simultaneously in plasma using reversed-phase ion-pairing chromatography with postcolumn sulfitolysis and reaction with 2-nitro-5-thiosulfobenzoate. The dynamic range of their assay for mesna and BNP7787 extended to only 67.5 μM, which is 1.8-fold lower than that of our assay for mesna and even 18-fold lower than that for BNP7787. The dynamic ranges of our assay in tissue were 20–2000 nmol/g for both mesna and BNP7787.

In plasma, mesna had a very short half-life[6] because of the fast formation of dimesna and mixed disulfides as well as rapid protein binding. However, in acid deproteinized plasma, mesna was stable for at least 24 h at room temperature.[6] Similar to our previously developed assay using HPLC with single ECD,[8] the mesna calibration and QC samples were prepared in acid deproteinized plasma instead of untreated plasma to obtain stable samples that could be easily and quickly prepared. The recovery of mesna from plasma compared to deproteinized plasma was almost complete and independent of the concentration. This result is comparable to the recovery obtained after using the same pretreatment procedure followed by HPLC with single ECD.[8] Thus, the mesna concentrations determined in patient plasma samples do not have to be corrected for loss during deproteinization. The recovery of mesna from deproteinized plasma, plasma, and tissue compared to the same concentration in an aqueous EDTA solution, all seemed to be concentration dependent. This concentration dependency of the mesna recovery could contribute to the nonlinearity of the calibration lines above the highest calibration sample. The recovery of BNP7787 from deproteinized plasma, plasma, and tissue was complete and independent of the concentration. The within- and between-day accuracies and precisions of mesna and BNP7787 in plasma and tissue were within the criteria for the validation of bioanalytical assays.[11]

Mesna and BNP7787 in tissue homogenate or deproteinized tissue homogenate prepared from the kidney were stable over the 12 weeks measured when stored at −80°C. At −20°C the stability of mesna decreased and was lower in tissue homogenate than in deproteinized tissue homogenate. Thus, although stabilizers of mesna (i.e., EDTA and sulfuric acid[8]) were present in the

tissue homogenate and in deproteinized tissue homogenate, loss of mesna could not be prevented. The increase of the BNP7787 concentration observed in deproteinized tissue homogenate during storage at −20°C could account for the loss of mesna.

With our assay, we could easily measure the plasma concentrations of mesna and BNP7787 in a patient receiving BNP7787 at 18.4 g/m^2 as a 15-min iv infusion during the first 6 h after administration. From the calculated half-lives of mesna and BNP7787 and the reported concentration–time data,[16,18–20] we may expect that we could even measure the plasma concentrations for up to ~24 h. This means that our assay can easily be used for pharmacokinetic purposes in patients.

Pendyala et al.[21,22] showed that mesna and BNP7787 administration can reduce the levels of endogenous thiols, such as glutathione and cysteine, in the circulation of patients. Thus, the possibility exists that mesna-mixed disulfides are formed in the circulation. Therefore, additional methods are needed to measure these mixed disulfides. With our single ECD assay,[8,9] we can additionally measure total mesna after reduction with sodium borohydride and then calculate the mixed disulfides concentration by [total mesna] − [mesna] − 2 × [BNP7787].

CONCLUSION

A sensitive, accurate, and precise method has been developed to determine mesna and BNP7787 simultaneously in small amounts of plasma and tissue by micro-HPLC using a dual amperometric electrochemical detector. This method allowed determination of the conditions for reliable storage of tissue samples containing BNP7787 and/or mesna. Furthermore, the assay was used to determine mesna and BNP7787 in plasma of a patient receiving BNP7787.

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