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Quantification of mesna and total mesna in kidney tissue by high-performance liquid chromatography with electrochemical detection

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Abstract

A sensitive and selective assay for the determination of mesna and total mesna in tissue was developed and validated. After a simple homogenization, extraction and deproteinization step, mesna could be measured immediately by HPLC with an electrochemical detector provided with a sensitive wall-jet gold electrode. Total mesna (i.e., free mesna and mesna present in mesna disulfides and mixed mesna disulfides) could be measured after pre-column reduction with sodium borohydride to free mesna. The lower limit of quantification of mesna and total mesna was for both compounds 10 nmol/g. The assays for mesna and total mesna in tissue were linear over the ranges of 10–3000 and 10–10 000 nmol/g, respectively. The within-day and between-day precisions of both methods were better than 9%. The within-day and between-day accuracy of the mesna assay ranged from 103.7 to 113.6%, whereas the accuracies of the total mesna assay ranged from 97.8 to 106.7%. Mesna in an EDTA containing tissue homogenate or in deproteinized tissue homogenate stored at −80 °C was stable for at least 12 weeks. Total mesna was stable under all conditions measured. The developed assays will be applied for the determination of the distribution of mesna and total mesna in tissues of the rat after administration of mesna or BNP7787.

Keywords: Mesna; BNP7787

1. Introduction

Mesna (sodium-2-mercaptoethanesulfonate) is an important thiol compound that prevents hemorrhagic cystitis in patients who receive oxazaphosphorine treatment, such as ifosfamide or cyclophosphamide, by neutralizing the highly reactive urotoxic metabolites of oxazaphosphorines locally in the urine [1–3]. It is also believed to be the only active metabolite of the disulfide BNP7787 (disodium 2,2′-dithio-bis-ethane sulfonate), which is under investigation as a non-toxic and new protector against cisplatin-induced toxicities especially nephrotoxicity [4]. After intravenous (i.v.) administration, BNP7787 is taken up in the kidney, intestine and liver and is enzymatically and locally converted into mesna (Fig. 1) [5–7]. The toxic (hydrated) cisplatin species, as the

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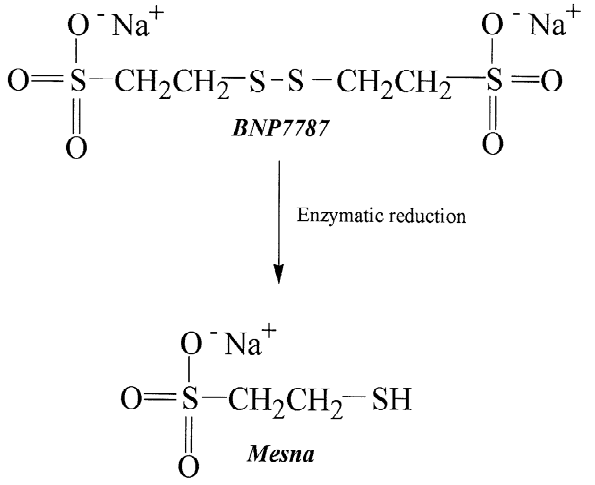

Fig. 1. Enzymatic reduction of BNP7787 to mesna in the kidneys, intestine, and liver.

urotoxic oxazaphosphorine metabolites, are rendered non-toxic by the formation of a complex with mesna.

A selective and sensitive method to determine mesna and total mesna (i.e., mesna and mesna present in dimesna (BNP7787) and mixed mesna disulfides originating from mesna reacted with endogenous thiols like glutathione and (homo)cysteine or sulfhydryl group containing proteins) in plasma and tissue is required to investigate the distribution and metabolism of (total) mesna after mesna or BNP7787 administration. A number of high-performance liquid chromatographic (HPLC) procedures have been used to separate mesna from endogenous thiols and other endogenous compounds present in biological fluids [8–13]. To quantify mesna in biological fluids, UV detection [8] is not sensitive enough unless pre-column [9] or post-column derivatization [11] of mesna takes place. Gatti et al. [14] have used a sensitive fluorogenic pre-column derivatization, which was only applied to pharmaceutical formulations. None of these methods determine the disulfides of mesna [9,11,14].

Up to now, the most frequently used and sensitive method to determine mesna and total mesna is HPLC with electrochemical detection [10–13]. In a previous report [10], we have described a sensitive and accurate method to determine mesna and total mesna in plasma and urine using HPLC with a sensitive electrochemical detector provided with a wall-jet gold electrode. Total mesna is measured indirectly as mesna after pre-column reduction with sodium borohydride. This method has been modified especially regarding sample treatment for the determination of both free mesna and total mesna in tissue. The developed assay appears to be a simple, selective, sensitive and accurate method. The method has been applied to determine the stability of total mesna and mesna in the kidney of a rat during storage under various conditions.

2. Materials and methods

2.1. Chemicals

Dimesna (BNP7787, disodium 2,2′-dithio-bis-ethane sulfonate) was provided by BioNumerik Pharmaceuticals (San Antonio, TX, USA) and mesna (sodium 2-mercaptoethanesulfonate) was obtained from Sigma (St. Louis, MO, USA). Trisodium citrate dihydrate, acetic acid, cysteamine chloride, *o*-phosphoric acid (85%), disodium hydrogenphosphate dihydrate and sulfuric acid were from Merck (Darmstadt, Germany). EDTA disodium salt, sodium borohydride and sodium hexametaphosphate were purchased from J.T. Baker (Deventer, The Netherlands). Tetrabutyl ammonium dihydrogenphosphate (1.0 *M* in water) was from Aldrich (Milwaukee, WI, USA) and sodium hydroxide was obtained from Riedel-de Haën (Seelze, Germany). Phosphate-buffered saline (PBS; pH 7.4, 10 m*M* sodium phosphate and 8.2 mg NaCl/ml) was from the hospital pharmacy VUMC, Amsterdam, The Netherlands. Deionized water from a Millipore Milli-Q system (Etten-Leur, The Netherlands) was used throughout this work.

2.2. Instrumentation

An isocratic HPLC system consisting of a Spark Triathlon 900 autosampler with a cooled tray (4 °C) (version 1.86), a Gynkotek M480 pump and a degasser Model GT-103 (all from Separations Analytical Instruments, H.I. Ambacht, The Netherlands) and a wall-jet electrochemical detector with gold working electrode set at an operating potential of +0.9 V relative to an Ag/AgCl reference electrode (Decade; Antec Leyden, Zoeterwoude, The Nether-

lands) were used. For the acquisition and processing of the data a Dionex Chromeleon Chromatography Data System (version 4.30) (Dionex Softron, Germering, Germany) was used.

2.3. Chromatography

Separation of mesna from endogenous compounds was performed on a Phenomenex Prodigy C_{18} ODS-3, 5 μm column (100×4.6 mm; Bester, Amstelveen, The Netherlands) preceded by a guard column with pellicular C_{18} refill (10 μm) (Alltech, Deerfield, IL, USA). The injection volume was 40 μl. The mobile phase consisted of an aqueous solution of trisodium citrate dihydrate (0.1 *M*), tetrabutyl ammonium dihydrogen phosphate (1.0 m*M*) and cysteamine (0.1 μ*M*), adjusted to pH 3.5 with 85% *o*-phosphoric acid. The flow-rate was set at 1 ml/min and the column temperature was kept at a temperature of 36 °C. The analysis time of one sample was 12.5 min.

2.4. Matrix

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

2.5. Calibration samples and quality control samples

Calibration samples of mesna and total mesna were prepared from two stock solutions in aqueous EDTA (1 g/l) containing 15 m*M* mesna or 10 m*M* dimesna (which is equivalent to 20 m*M* total mesna). The same solutions were independently prepared for the preparation of the quality control samples. These solutions were kept at 4 °C and were stable for at least 2 months at 4 °C [13].

2.5.1. Mesna calibration and quality control samples

The mesna stock solution was diluted with aqueous EDTA (1 g/l) to obtain an appropriate range of calibration solutions. Tissue homogenate (190 μl) was spiked with 10 μl of the calibration solutions to obtain mesna calibration samples of 10, 30, 100, 300, 1000 and 3000 nmol/g. The calibration samples were prepared freshly, on ice and in duplicate. The mesna quality control samples of 15, 150 and 1500 nmol/g were prepared in the same way using the other mesna stock solution. After spiking, the samples were immediately deproteinized by adding one volume of cold 0.33 *M* sulfuric acid and one volume of cold sodium hexametaphosphate (5 g/100 ml) to one volume of calibration or quality control sample. After vortexing well and centrifugation for 2 min at 9000 rpm, 400 μl of supernatant was added to 80 μl of 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 in order to prevent fast deterioration of the analytical column. The samples were ready to be injected into the HPLC system.

2.5.2. Total mesna calibration and quality control samples

The calibration samples of total mesna were prepared freshly, on ice and in duplicate. The dimesna stock solution was diluted with aqueous EDTA (1 g/l) to obtain different dimesna calibration concentrations. Tissue homogenate (190 μl) was spiked with 10 μl of the calibration solutions to obtain the following calibration samples: 5, 10, 50, 100, 500, 1000 and 5000 nmol/g dimesna in tissue. The total mesna quality control samples were also prepared by spiking tissue homogenate with an other dimesna stock solution. This resulted in quality control samples of 25, 250 and 2500 nmol/g dimesna in tissue. (The corresponding total mesna concentrations of the calibration and quality control samples which will be obtained after reduction with sodium borohydride will be two times higher). After spiking the tissue homogenate, one volume of calibration or quality control sample was immediately deproteinized by adding one volume of cold 0.33 *M* sulfuric acid and one volume of cold sodium hexametaphosphate (5 g/100 ml). The mixture was vortexed well and centrifuged for 2 min at 9000 rpm. Total mesna was quantified as mesna after reduction of dimesna with sodium borohydride. For that purpose 100 μl deproteinized tissue homogenate 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 vortexed 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 column.

2.6. Assay optimization

2.6.1. Hydrodynamic voltammogram

The optimal oxidation potential for the detection of mesna was determined by measuring a hydrodynamic voltammogram. Samples of 100 μ*M* mesna in mobile phase and 1052 nmol/g mesna and 50 nmol/g dimesna (after reduction with sodium borohydride) in tissue were measured by the described HPLC system at potentials from +0.4 V up to +1.0 V with increments of 0.1 V. The obtained peak heights of mesna and the background current were plotted against the working potential. The optimal oxidation potential was obtained at the highest ratio of the peak height of mesna to the background current.

2.6.2. Sample stability during and after processing

The stability of mesna during sample processing was established by incubating 72.5 nmol/g mesna in quadruplicate in aqueous EDTA (1 g/l), tissue homogenate (containing 1 g/l EDTA) or deproteinized tissue homogenate at 0 °C, room temperature (22 °C), or 37 °C. The mean peak height of the samples measured immediately after preparation were set at 100%.

Stability of mesna and total mesna in the autosampler of 4 °C was investigated by analyzing a series of quality control samples over 24 h.

2.7. Calculations

Calibration samples were analyzed in duplicate. The mean of the peak heights at each calibration level was used to calculate the linear regression parameters using a weighting factor $1/x$ for both assays. The concentrations of the quality control and stability samples were calculated by interpolation of the peak heights of the quality control and stability samples on the calibration line.

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

2.8. Assay validation

A validation of the assays for mesna and total mesna in tissue was performed on the following parameters: selectivity, recovery, lower limit of quantification (LLQ), linearity, within-day and between-day accuracy, within-day and between-day precision and stability.

2.8.1. Selectivity

Retention times of the endogenous thiols glutathione, cysteine, homocysteine and methionine were determined to check whether these compounds interfered with the retention times of mesna.

2.8.2. Recovery of mesna and total mesna from tissue

The recoveries of mesna and total mesna from tissue were determined by spiking blank tissue homogenate (in sixfold) or blank EDTA (1 g/l) (in sixfold) with mesna or dimesna to obtain samples with mesna concentrations of 15, 150 and 1500 nmol/g or total mesna concentrations of 50, 500 and 5000 nmol/g. The tissue and EDTA samples were immediately deproteinized and further treated as described before. The recovery at each concentration level was calculated by expressing the mean peak height obtained for the tissue samples as percentage of the mean peak height obtained for samples with the same mesna or dimesna concentration in the aqueous EDTA solution.

2.8.3. Lower limit of quantification

The LLQ was the lowest concentration of mesna or total mesna which 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 concentrations of calibration samples in the lowest concentration range.

2.8.4. Linearity

A calibration line was considered linear when the correlation coefficient (r^2) was higher than 0.99, when the accuracy and precision of the calibration samples were less than 15% with exception of the LLQ for which an error of maximal 20% was excepted and when the signs of the residuals were randomly distributed over the whole concentration range [15]. The linearity of the calibration curves was also determined with the *F*-test for lack of fit (F_{LOF}) as described by Massart et al. [16]. The sum of squared residuals after linear regression was compared to the sum of squares due to purely experimental uncertainty (variability within each group of replicate measurements) (both calculated by analysis of variance) with an *F*-test (F_{LOF}) using a *P*-value of 0.05. Linearity was obtained when the F_{LOF} value was lower than the tabulated critical value.

Student's *t*-test was applied to the following linear model [15,17] to test if the assay was subjected to translational or rotational bias:

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

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* 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:

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

in which S.E. is the standard error of *a* or *b*. 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.

2.8.5. 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 quality control samples of mesna and dimesna in sixfold on 1 day, whereas the between-day analysis was done in duplicate on 6 different days. On each day a complete set of calibration samples was also analyzed in duplicate.

2.8.6. Stability of mesna and total mesna in tissue

The stability of mesna and total mesna in the kidney of a rat was determined during storage. A rat was injected i.v. with 1000 mg/kg BNP7787. Forty-five minutes after the BNP7787 administration the kidneys were removed and immediately frozen after washing with a cold PBS solution to remove the blood. The frozen kidneys were transferred to the laboratory as soon as possible where they were pulverized with a micro-dismembrator. An appropriate volume of aqueous EDTA (1 g/l) was added to obtain a solution of 100 mg/ml (wet mass). After 10 min of sonicating and 10 min of vortexing, part of the kidney homogenate sample was stored in aliquots at −20 and −80 °C. The remaining part of the kidney homogenate sample was immediately deproteinized by adding one volume of cold 0.33 *M* sulfuric acid, one volume of cold sodium hexametaphosphate (5 g/100 ml) and 1/20 volume of aqueous EDTA (1 g/l) (to correct for spiking of calibration and quality control samples) to 19/20 volume of kidney homogenate sample. After vortexing well and centrifugation for 2 min at 9000 rpm, the supernatant of the deproteinized kidney sample was stored in aliquots at −20 and −80 °C until analysis. The stability in the different tissue matrices was determined by analyzing mesna and total mesna concentrations in duplicate immediately after removal (day 0) and at various time points thereafter up to 12 weeks of storage. The stability sample was considered stable when the concentration differed less than 15% from the concentration at the start.

3. Results and discussion

3.1. Assay optimization

Our assay developed for the analysis of mesna and total mesna in plasma and urine [10] was used as a starting point to analyze these compounds in tissue. Tissue samples were analyzed under plasma conditions. No interfering peaks were observed at the

retention time of mesna when comparing a chromatogram of a deproteinized pig kidney sample spiked with mesna with that of a blank deproteinized pig kidney sample (Fig. 2). Likewise, a deproteinized pig kidney homogenate did not show any interfering peak at the retention time of mesna after reduction with sodium borohydride (Fig. 3). The chromatograms of deproteinized rat kidney homogenate were comparable to those of deproteinized pig kidney homogenate. These results show that the chromatographic conditions used for the analysis of (total) mesna in plasma [10] could also be used for the analysis of (total) mesna in tissue.

The optimal oxidation potential of the gold working electrode was determined by a hydrodynamic voltammogram of mesna in mobile phase. This voltammogram showed a maximum mesna to background current ratio at a potential of +0.9 V vs. Ag/AgCl. The effect of the matrix was investigated by measuring a hydrodynamic voltammogram of mesna in deproteinized tissue homogenate instead of mobile phase (Fig. 4). The voltammogram in the deproteinized tissue homogenate showed almost the same response curve and background current as the voltammogram of mesna in mobile phase. Hence, the tissue matrix did not affect the optimal oxidation potential. Furthermore, a hydrodynamic voltammogram of dimesna in deproteinized tissue homogenate measured after reduction to mesna (i.e., total mesna) with sodium borohydride also showed that the

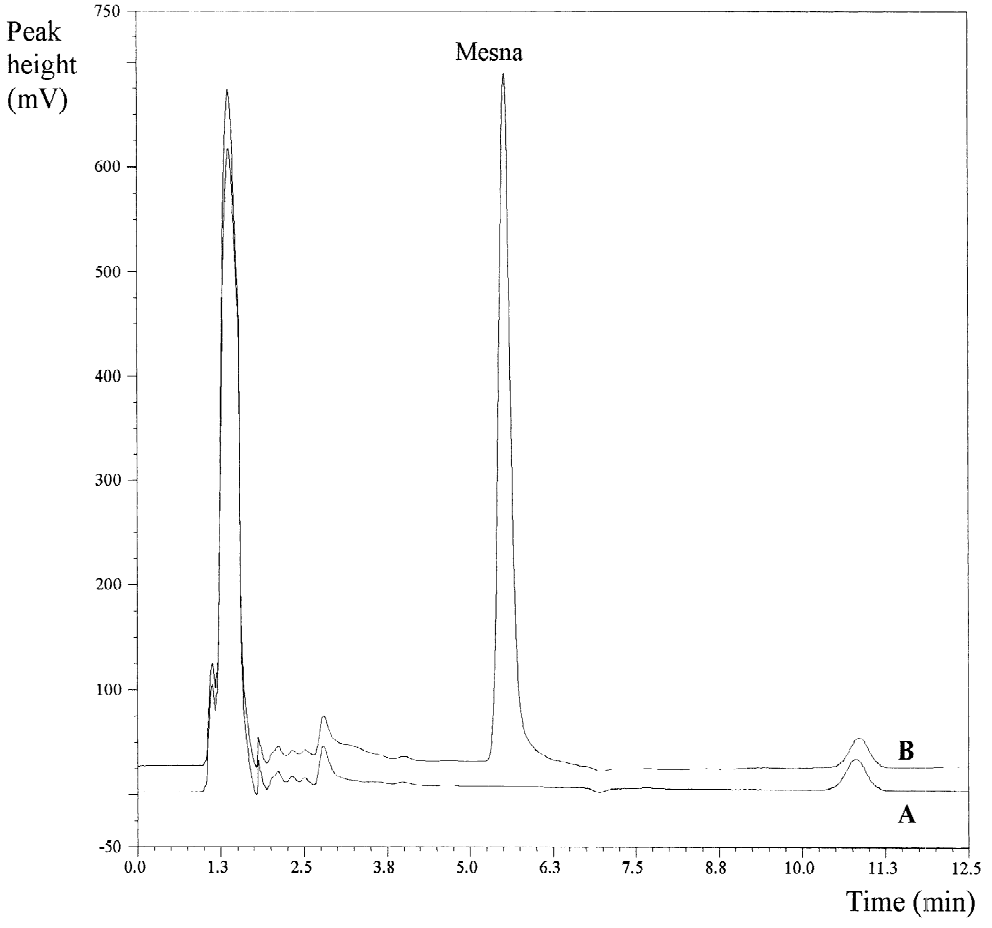

Fig. 2. A representative chromatogram of blank deproteinized pig kidney homogenate (A) spiked with 1000 nmol/g mesna (B).

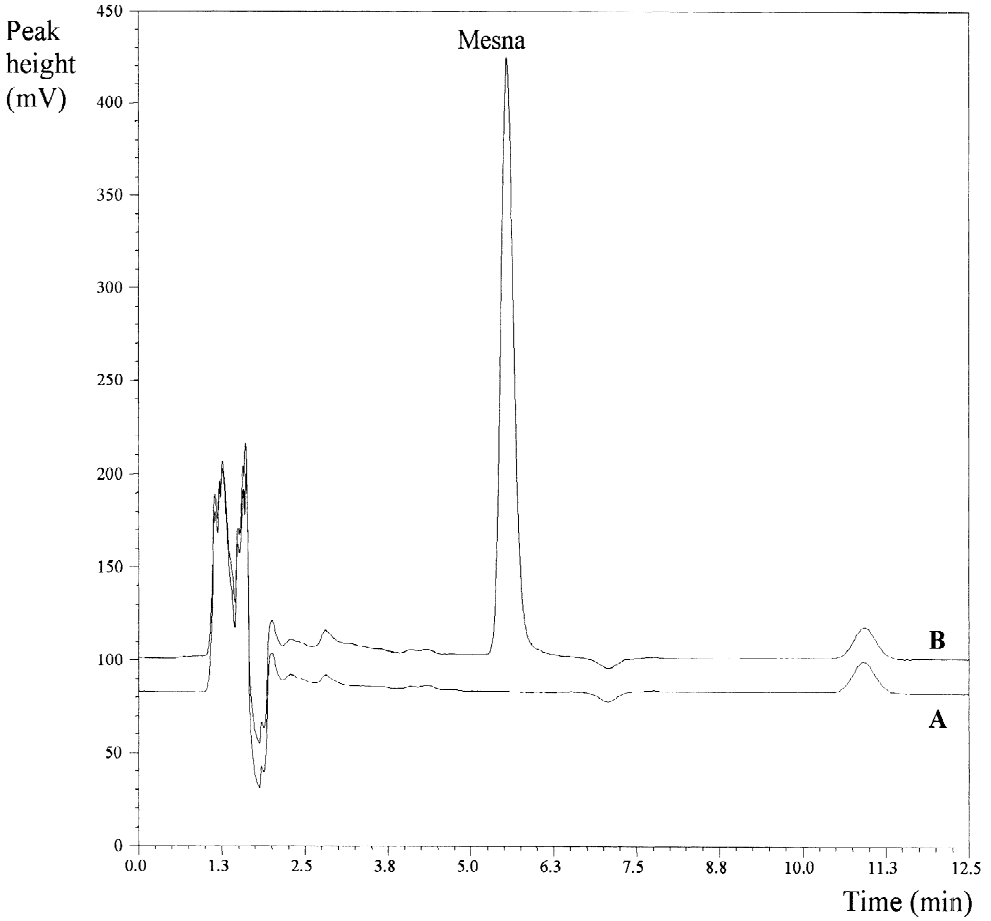

Fig. 3. A representative chromatogram of blank deproteinized pig kidney homogenate (A) spiked with 1000 nmol/g dimesna (B) both measured after reduction with sodium borohydride.

optimal oxidation potential was +0.9 V vs. Ag/AgCl.

The thiol mesna is more reactive and thus less stable than its disulfide dimesna [10,18]. Therefore, we investigated only the stability of mesna during sample processing. Mesna was stable for at least 30 min in aqueous EDTA and in deproteinized tissue homogenate at 37 and 0 °C. The stability of mesna in tissue homogenate containing EDTA (1 g/l), however, showed that after 30 min of incubation at 37 °C, room temperature and 0 °C the recovery of mesna decreased by approximately 57, 40 and 19%, respectively. Thus, to minimize the loss of mesna, the preparation of the calibration and quality control samples in tissue homogenate has to be done on ice. Furthermore, the samples have to be deproteinized as quickly as possible after spiking with mesna which is similar to the treatment of blood samples [10,13].

3.2. Assay validation

3.2.1. Selectivity

Under the conditions used the retention times of the main endogenous thiols glutathione, cysteine, homocysteine and methionine were at least 2 min shorter than the retention time of mesna ($t_R = 5.4$ min) originating from tissue. Thus, no interference might be expected by possibly changing concen-

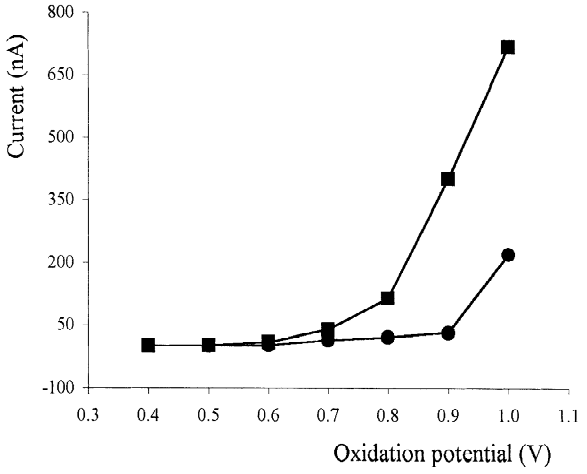

Fig. 4. Hydrodynamic voltammogram of the peak height of 100 μ*M* mesna in deproteinized pig kidney homogenate (■) and the background current (●).

trations of these compounds due to treatment with (di)mesna [19,20].

3.2.2. Recovery of mesna and total mesna from tissue

The recovery of mesna from tissue ranged from 85.3 to 91.7% at the three concentration levels of the quality control samples and seemed to be concentration dependent (Table 1). The recovery of total mesna, which ranged from 68.3 to 79.9%, was lower than the recovery of mesna and also concentration dependent (Table 1).

3.2.3. Lower limit of quantification

The LLQ for mesna was 10 nmol/g tissue. The assay for total mesna had the same LLQ (i.e., 10 nmol/g tissue) indicating that both assays were sensitive. Taking into account the dilutions of the tissue samples and the 40 μl injection loop, the LLQs of mesna and total mesna corresponded with injected amounts of 10.6 and 2.6 pmol, respectively. The endogenous thiol glutathione has also been determined in tissue using HPLC with electrochemical detection [21–23]. The LLQ of mesna was comparable to the reported LLQs of glutathione [21–23].

Table 1
Mean recoveries (±SD) of mesna and total mesna from tissue

Analyte	Tissue	
	Concentration (nmol/g)	Recovery±SD (%)
Mesna	15	85.3±1.9
	150	87.2±2.8
	1500	91.7±4.5
Total mesna	50	68.3±5.0
	500	74.8±8.0
	5000	79.9±9.2

3.2.4. Linearity and dynamic range

The linearity of the mesna assay ranged from 10 to 3000 nmol/g. The dynamic range for the total mesna assay was even broader, i.e., 10–10 000 nmol/g. For both assays the correlation coefficients of the six calibration lines were better than 0.994. The accuracy and precision of the mesna and total mesna calibration samples were smaller than 15% over the whole concentration range and smaller than 20% at the LLQ. Almost all calibration lines of mesna (four out of six) and total mesna (five out of six) proved to be linear with the *F*-test for lack of fit. The calibration lines, which were non-linear according to the lack of fit test, had small variances in the replicates of the calibration samples. The t_a and t_b values of all calibration lines for mesna and total mesna were less than the tabulated critical values, indicating that no significant translational and rotational bias could be detected.

3.2.5. Within- and between-day accuracy and precision

The within- and between-day accuracy and precision of mesna and total mesna analyzed in the quality control samples prepared in deproteinized tissue homogenate are summarized in Table 2. For the mesna analysis, the within-day and between-day precision of the quality control samples were better than 6 and 9%, respectively. The within-day and between-day accuracies of these samples were within 13.6 and 5.2% of the nominal values, respectively. The within-day and between-day accuracy of total mesna was within 6.7% of the nominal values. The within-day and between-day precision for total mesna were both better than 7%. Both accuracy and precision were within the criteria [15].

Table 2
Within-day and between-day accuracy and precision of the three quality control samples of mesna and total mesna in deprotenized tissue homogenate

Analyte	Concentration (nmol/g)	Accuracy (%)		Precision (%)	
		Within-day ($n=6$)	Between-day ($n=6$)	Within-day ($n=6$)	Between-day ($n=6$)
Mesna	15	113.6	104.7	2.7	9.0
	150	111.3	105.2	2.7	6.6
	1500	104.9	103.7	5.7	4.2
Total mesna	50	97.8	98.5	6.5	2.2
	500	97.8	104.7	2.7	4.6
	5000	103.5	106.7	1.2	2.7

3.3. Stability of mesna and total mesna during storage

The developed assays of mesna and total mesna were applied to determine the stability of both analytes in the kidney of a rat during storage. Forty-five min after an i.v. injection of 1000 mg/kg BNP7787 the kidneys were removed from the rat and stored under various conditions. Immediate analysis showed that the concentration of mesna (7.6 μmol/g) in the kidney was approximately 64% of the concentration of total mesna (11.8 μmol/g). This means that the difference between total mesna and free mesna represents mesna present as the symmetrical disulfide of mesna (dimesna) and/or as mixed mesna disulfides. Because the concentrations of mesna and total mesna were (just) above the dynamic range of our assays, the samples were measured after an appropriate dilution with blank tissue homogenate or deproteinized tissue homogenate.

Mesna and total mesna in tissue homogenate containing EDTA (1 g/l) and in deproteinized tissue homogenate stored at −80 °C were stable over the 12 weeks measured. However, stored at −20 °C, the mesna concentration in tissue homogenate decreased with 96% in 2 weeks, whereas total mesna remained unchanged. Thus, although EDTA was present in the tissue homogenate, it could not prevent the loss of mesna by the formation of disulfides (i.e., dimesna and/or mixed mesna disulfides) at −20 °C. The mesna concentration in deproteinized tissue homogenate stored at −20 °C showed a smaller decrease in time compared (67% left after 2 weeks of storage, $t_{1/2,obs}=28$ days). Thus, deproteinizing the tissue homogenate with sulfuric acid and hexametaphosphate increased the stability of mesna. The total mesna concentration in the deproteinized tissue samples stored at −20 °C remained unchanged. Thus, tissue samples not immediately analyzed have to be deproteinized and stored at −80 °C.

4. Conclusion

The described HPLC assays using a sensitive electrochemical detector provided with a wall-jet gold electrode are suitable to measure mesna and total mesna in tissue. These methods are sensitive, accurate, precise and simple and meet the requirements for validated bioanalytical assays. Tissue samples, which cannot be analyzed immediately, should be dismembrated, deproteinized and stored at −80 °C. The presented methods will be implemented in the determination of the pharmacokinetics of mesna and BNP7787 in rats.

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