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Talanta 66 (2005) 534–539
Talanta
www.elsevier.com/locate/talanta

Urinary mesna and total mesna measurement by high performance liquid chromatography with ultraviolet detection

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Received 9 April 2004; received in revised form 23 September 2004; accepted 16 November 2004
Available online 1 January 2005

Abstract

We describe in this report a method for determination of mesna and total mesna in urine by high performance liquid chromatography with ultraviolet detection. The method involves a treatment of the urine sample with tri-*n*-butylphosphine in order to convert mesna disulfides to its reduced counterpart mesna, ultraviolet labelling with 2-chloro-1-methylquinoluinium tetrafluoroborate, reversed-phase HPLC separation, and detection and quantitation at 350 nm. The result corresponds to total mesna that is sum of mesna, dimesna and its mixed disulfides with endogenous thiols. For determination of mesna the reduction step is omitted. Content of disulfide forms of mesna can be calculated by subtracting the concentration of mesna from the total mesna concentration. The separation of 2-*S*-quinolinium derivatives of mesna from those of endogenous urinary thiols and internal standard was achieved on an analytical Waters Nova-Pak C18 (150 mm × 3.9 mm, 5 μm) column. A mixture of an aqueous solution of pH 2.3, 0.05 M trichloroacetic acid and acetonitrile (88:12, v/v) was used as a mobile phase at flow rate of 1.2 ml/min and ambient temperature.

The assay for mesna and total mesna in urine was proved to be linear over the studied ranges of 0.2–30 and 0.2–800 nmol/ml urine, respectively. The mean recoveries over the calibration ranges were 95.4% for mesna and 99.7% for total mesna. The lower limits of detection and quantitation were 0.1 and 0.2 nmol/ml for both the procedures, respectively. The imprecision did not exceed 8.5%. No interference from endogenous substances was observed.

Keywords: Mesna; Total mesna; Urine; HPLC; Derivatization

1. Introduction

Numerous studies have demonstrated that sulfhydryl-containing nucleophiles like *N*-acetylcysteine and mesna (2-mercaptoethanesulfonate) can antagonize the dose-limiting effects of alkylating anticancer agents on the genitourinary tract. The superiority of mesna as a chemoprotector to other available thiols has been confirmed clinically in an ifosfamide trial [1]. The urotoxic oxazaphosphorine metabolites are detoxified by their reactions with the sulfhydryl group of mesna [2]. Mesna does not block the antitumor action of oxazaphosphorines most likely due to its rapid formation of the inactive dimer dimesna in the blood-stream [3]. The active thiol monomer is reformed by reduction of dimesna in renal tubule cells, thereby inactivating toxins like acrolein to the bladder. In spite of routine use of mesna in patients receiving high doses of oxazaphosphorines, hemorrhagic cystitis occurs in some cases. This may be ascribed, among others, to insufficient urinary concentration of mesna [4]. In order to ensure constant protection of genitourinary tract sufficient levels of free thiol must be maintained during the period when the toxic metabolites are excreted. Monitoring the urinary levels of mesna, is therefore, clinically important.

Apart from non-specific and not very sensitive colorimetric, based on Ellman's reagent, methods [5,6] several HPLC procedures for determination of mesna in biological samples [4,7–12] have been elaborated. Ultraviolet detection is not

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doi:10.1016/j.talanta.2004.11.029

enough sensitive for this purpose unless pre-column [8] or post-column [10] derivatization is applied. A majority of the HPLC procedures take advantage of electrochemical detection [9–12].

In a previous paper [8], we have reported a sensitive and accurate method for determination of mesna in plasma with the use of standard HPLC system equipped with by far the most popular UV–vis detector. The method measures mesna in the form of its 2-*S*-quinolinium derivative after pre-column derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate a thiol specific UV-tagging reagent [13]. Below we describe an extension of this method to cover mesna and its oxidized forms in urine. Total mesna is measured as mesna after pre-column treatment with tri-*n*-butylphosphine.

2. Experimental

2.1. Apparatus and instruments

HPLC analyses were performed with a Hewlett–Packard (Waldbronn, Germany) HP 1100 Series system equipped with quaternary pump, an autosampler, thermostated column compartment, vacuum degasser and diode-array detector and controlled by HP ChemStation software. UV spectra were recorded on a Hewlett–Packard HP 8453 (Waldbronn, Germany) diode-array UV–vis spectrophotometer. Water was purified using a Millipore Milli-QRG system (Vien, Austria). For pH measurement, a Hach One (Loveland, USA) pH meter was used.

2.2. Reagents and solutions

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory as described in our previous report [13]. 2-Mercaptoethanesulfonic acid, sodium salt (mesna, MES) and 3-mercaptopropane sulfonic acid, sodium salt (MPS) was purchased from Aldrich Europe (Beerse, Belgium). Dimesna $(MES)_2$ and 3,3′-dithiodipropanesulfonic acid, disodium salt $(MPS)_2$ were prepared in this laboratory. Thiomalic acid (TMA), tri-*n*-butylphosphine (TNBP), 2-mercaptopropionic acid (2MPA), 3-mercaptopropionic acid (3 MPA) and thioglicolic acid (TGA) were from Fluka (Buchs Switzerland), ethylenediaminetetraacetic acid, disodium salt (EDTA), perchloric acid (PCA), HPLC-grade acetonitrile (ACN) and methanol (MeOH) were provided by J.T. Baker (Deventer, The Netherlands). Tris(hydroxymethyl)aminomethane (Tris) and trichloroacetic acid (TCA) were from Merck (Darmstadt, Germany). All other chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

TCA and Tris buffer solutions of appropriate concentration were adjusted to the desired pH by potentiometric titration with lithium hydroxide and hydrogen chloride solution, respectively. The titration systems were calibrated with standard pH solutions. Standard thiol solutions (10 μmol/ml) were prepared in water or dilute hydrochloric acid, standardized with *o*-hydroxymercurybenzoate [14] and kept at 4 °C. The working solutions were prepared daily as needed.

2.3. Chromatography

An autosampler handed 20-μl volume of the final analytical solution onto a Nova-Pack C18 column (150 mm × 3.9 mm, Waters, USA) packed with 5 μm particles. Mobile phase consisted of 0.05 M TCA buffer, adjusted to pH 2.3 with lithium hydroxide solution of the same concentration, and acetonitrile in ratio of 88:12 (v/v). The flow rate was 1.2 ml/min, the temperature 25 °C and the detector wavelength 350 nm. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at real time of analysis, with corresponding set of data obtained with authentic compounds.

2.4. Calibration standards

Stock solutions of 10 μmol/ml mesna, dimesna and internal standards sodium 3-mercaptopropanesulfonate and disodium 3,3′-dithiodipropanesulfonate were prepared by dissolving an appropriate amounts of each compound in 2 ml of 0.1 M hydrochloric acid and diluting to the volume of 10 ml. These solutions were kept at 4 °C for several days without noticeable change in the solutes concentrations. The working solutions were prepared by appropriate dilutions with water as needed. For preparation of calibration standards for total mesna (dimesna) in urine, portions of 0.5 ml of urine from apparently healthy donors were each placed in a sample tube and spiked with increasing amounts of working standard solution of dimesna. Assuming 100% of the future reduction of the disulfide bonds, concentration of mesna in the consecutive tubes was 0.2, 0.5, 2.0, 8.0, 15.0, 30.0, 100.0, 400.0, 600.0 and 800.0 nmol/ml urine. For mesna the same amount of urine was spiked with standard mesna solution giving concentration of 0.2, 0.5, 2.0, 6.0, 8.0, 15.0 and 30 nmol/ml urine.

2.5. Search for internal standard

Several thiols such as 2- and 3-mercaptopropionic acid, thioglycolic acid, thiomalic acid and 3-mercaptopropanesulfonic acid and their disulfides (in concentration 10 nmol/ml) were added first to mesna water standard and next to urine spiked with mesna or dimesna and the resulted mixtures were subjected to all steps of the appropriate analytical procedure. *N*-Acetylcysteine, *N*-(2-mercaptopropionyl)glycine and penicillamine were not taken into consideration because they can be present as drugs.

2.6. Analytical procedures

2.6.1. Determination of total mesna, procedure 1

To 500 μl of urine 0.25 ml of 0.1 M EDTA, 0.5 ml of 1 M, pH 7.3 Tris buffer, 75 μl of 0.1 μmol/ml $(MPS)_2$ (internal

standard), and 25 μl of 10% tri-*n*-butylphosphine in methanol were added. The mixture was incubated for 30 min at 60 °C, and after cooling on ice water 50 μl of 100 μmol/ml CMQT derivatization reagent was added and the mixture was put aside for 5 min followed by acidification with 300 μl of 3 M PCA and centrifugation for 5 min. A 20-μl aliquot of supernatant was transferred into HPLC system.

2.6.2. Determination of mesna, procedure 2

The urine sample 500 μl containing internal standard (MPS, 15 nmol/ml urine) was derivatized with 50 μl of 100 μmol/ml CMQT in the presence of 0.25 ml 0.1 M EDTA and 0.5 ml of 1 M pH 7.3 Tris buffer, and further processed according to procedure 1.

2.6.3. Calibration curves

The urine calibration standards for total mesna and mesna within the concentration ranges from 0.2 to 800 and 0.2 to 30 nmol/ml were processed according to procedures 1 and 2, respectively. The peak height ratios of mesna-CMQT derivatives to that of internal standard were plotted versus analyte concentrations and the curves were fitted by least-square linear regression analysis.

2.7. Assay validation

2.7.1. Selectivity

Retention times of the endogenous urine thiols were determined to check whether these compounds, known to react with CMQT [13,15], interfered with the retention times of mesna and internal standard CMQT derivatives.

2.7.2. Lower limits of detection and quantitation

The limits of detection were assessed as the minimum detectable concentration of mesna that could be detected without interference from the baseline noise (signal-to-noise ratio of 3:1). The limit of quantitation was determined as the minimum concentration of which, both, inaccuracy and imprecision are within 20%.

2.7.3. Linearity of the assay

The linearity between the concentration of mesna and dimesna and the peak height ratios of respective analyte-CMQT derivative to that of internal standard was determined by analyzing normal urine spiked with the standard solutions of analytes as described in Section 2.4. The linearity was demonstrated using 7–10-point calibration curves, and at each concentration four replicates were assayed, independently for mesna and oxidized mesna according to the recommended analytical procedures (Section 2.6).

2.7.4. Imprecision, inaccuracy and recovery

Intra-run imprecision, inaccuracy and recovery were determined in conjunction with the linearity studies for four concentrations. Known concentrations of mesna or dimesna along with the internal standard were added to normal mesna-free urine and the samples were processed according to the recommended appropriate analytical procedure. Four concentrations were studied: two near the lower limit of quantitation, one near the center and one near the upper boundary of the calibration curve. Measured concentrations were assessed by application of calibration curve obtained on that occasion. Imprecision was expressed in terms of the relative standard deviation (R.S.D.), while inaccuracy was determined from the mean relative error (E_{rel}, i.e., difference between measured and nominal concentrations of the spiked samples) in a replicate set.

Recovery at each concentration levels was calculated by expressing the mean measured amount as percentage of added amount.

3. Results and discussion

3.1. Derivatization

Mesna, similarly to majority of hydrophilic thiols, does not show the structural properties necessary for the production of signals compatible with the most common HPLC detectors, that is fluorescence and ultraviolet absorbance detector. Therefore, for signal enhancement, and at the same time labile sulfhydryl group blocking, several derivatization reagents have been described in the literature (for review see ref. [16]). In this work for derivatization of mesna we have applied 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT). The reaction between CMQT and thiols including mesna was described in details earlier [8,13]. Briefly, mesna and its close homolog sodium 3-mercaptopropanesulfonate (internal standard) react quantitatively in urine at the presence of slightly alkaline buffer to give corresponding 2-quinolinium derivatives (Fig. 1). These stable thioethers show a well-defined absorption maximum at 350 nm.

3.2. HPLC separation conditions

In order to establish optimum chromatographic conditions for separation of CMQT derivatives of mesna, internal standard, endogenous urinary thiols and unidentified matrix components we have investigated the influence of several parameters. The influence of mobile phase organic modifier content, TCA buffer pH and concentration on resolution, retention factors and peak heights was studied.

As an organic modifier in the mobile phase we have tried acetonitrile and methanol. From Fig. 2 can be seen that the peaks were two to four folds higher with acetonitrile as compared with methanol. These solvents are known to be sorbed at the surface of the column packing material, and are in competing equilibrium with lipophilic moieties of analytes for absorption sites of the chemically bound stationary phase. Unlike acetonitrile, methanol forms hydrogen bonds resulting in increase of the retention times,

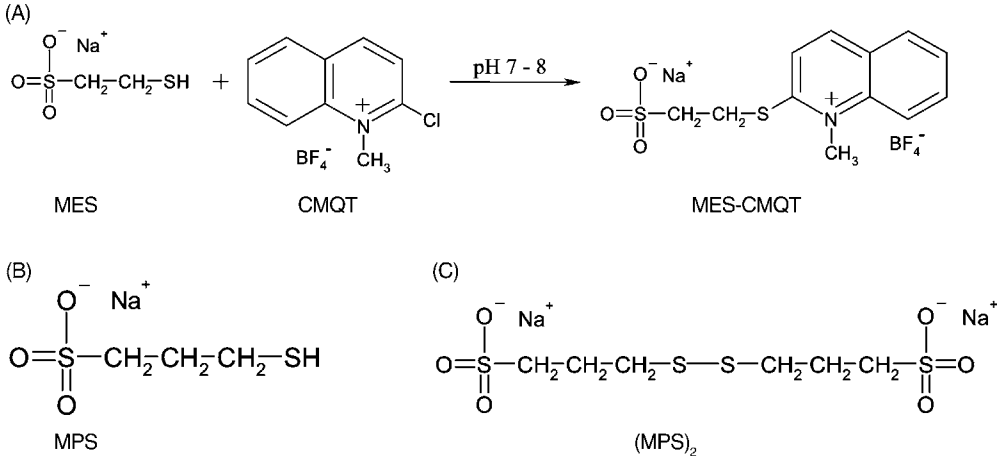

Fig. 1. Chemical derivatization reaction equation of MES with CMQT (A), and chemical structures of the internal standards for determination of MES (B), (sodium 3-mercaptopropanesulfonate, MPS) and total mesna (C) (sodium 3,3′-dithiodipropanesulfonate $(MPS)_2$).

decrease of peak heights, and higher back pressure at an analytical column. Therefore, acetonitrile was chosen based on the shorter retention times and better peaks shape of the analytes.

TCA buffer pH did not have much effect on the retention factors of mesna and internal standard CMQT derivatives due to high similarity in chemical structures. In the pH range investigated, from 2 to 4.5, the sulfonic acid group is equally ionized in both compounds, and as a consequence their CMQT derivatives receive very similar charge. As a result of the permanent positive charge on quaternary nitrogen atom in the quinolinium moiety they are practically zwitterionic. Similar charge means similar interaction with the TCA pairing agent, and similar retention (data not shown). Based on the above, briefly mentioned experimental results we have chosen optimum conditions for HPLC separation, specified in Section 2.3, under which CMQT derivatives of mesna and internal standard eluted after 1.24 (R.S.D. 0.44%; $n = 6$) and 1.41 (R.S.D. 0.58%; $n = 6$) min, respectively. Both peaks are well separated from each other and urine matrix components. Typical chromatograms for urine blank and the urine spiked with mesna and internal standard are shown in Fig. 3.

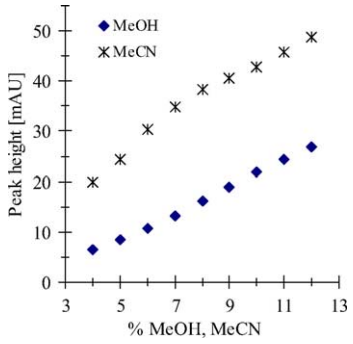

Fig. 2. Effect of the mobile phase organic modifier content on mesna-CMQT peak height.

3.3. Selectivity

The retention times of the main urinary thiols cysteine and homocysteine CMQT derivatives, under the recommended conditions, were at least 3 min longer than that of mesna. Thus, no interference might be expected even in the case of dramatical increase in excretion of these aminothiols due to treatment with uroprotector mesna (or dimesna) [17,18].

3.4. Linearity

The calibration curves were linear in the range from 0.2 to 30 nmol/ml and 0.2 to 800 nmol/ml urine for mesna and its oxidized form dimesna, respectively. The equations for the linear regression and coefficients of correlation were $y = 0.0728x + 0.016$, $R^2 = 0.9947$, and $y = 0.0019x + 0.0142$, $R^2 = 0.9972$, respectively. The corresponding set of data obtained using peak area ratios was $y = 0.0253 + 0.0416$, $R^2 = 0.9974$ and $y = 0.0012x + 0.0006$, $R^2 = 0.9988$, respectively. The calibration ranges can be extended up if needed.

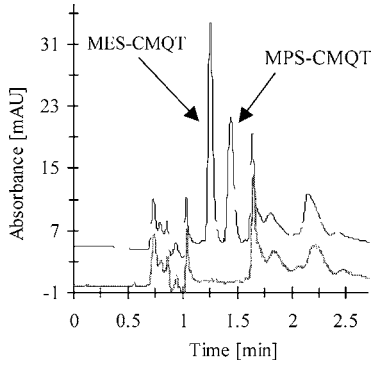

Fig. 3. A representative chromatogram of blank urine (gray lane) spiked with 20 nmol/ml dimesna and 15 nmol/ml internal standard $(MPS)_2$ (black line) measured after analytical procedure 1 as described in Section 2.6.1.

Table 1
Recovery, imprecision and inaccuracy for urinary mesna and total mesna determination by HPLC-UV method ($n = 5$)

Nominal concentration (nmol/ml)	Recovery (%)	Imprecision (R.S.D., %)	Inaccuracy (E_{rel}, %)
Mesna			
0.2	93.2	7.6	−6.8
8.0	90.3	3.6	−10
15.0	101.5	2.7	1.5
30.0	96.7	3.2	−3.3
Total mesna (dimesna)			
0.2	96.1	8.5	−3.9
2.0	98.3	5.3	−1.7
400.0	101.0	3.2	4.7
800.0	103.4	2.9	1.4

3.5. Lower limits of detection and quantitation

The lower limits of detection and quantitation of mesna and total mesna with 20-μl injection were both 0.1 and 0.2 μmol/ml urine, respectively. At concentration corresponding to lower limit of quantitation the imprecision and inaccuracy were for mesna 7.6 and 6.8% and for dimesna 8.5 and 3.9%, respectively. The concentration sensitivity of the assay can be increased by increasing the injection volume.

3.6. Imprecision, inaccuracy and recovery

Intra-run imprecision and inaccuracy for the determination of mesna and total mesna—expressed in R.S.D. values and mean relative error, respectively—were determined for four concentrations representing the whole range of each calibration curve. The imprecision and inaccuracy were for mesna within 7.6–2.7% and 6.8–1.5%; and for total mesna 8.5–2.9%, and 3.9–1.4%, respectively. Recovery values were from 93.2 to 101.5% and from 96.1 to 103.4%, respectively. Detailed data are in Table 1.

4. Conclusion

Mesna and total mesna in urine can be easily and reliably determined by CMQT derivatization HPLC-UV method. The content of oxidized forms of mesna, that is sum of dimesna and mesna mixed disulfides with urinary endogenous thiols such as cysteine or homocysteine, are assessed by subtraction of the result for mesna from that of total mesna. The method does not differentiate between dimesna and mixed disulfides of mesna. Analytical figures of merit demonstrated during the method validation protocol compare well with those of known methods [4–12] for determination of mesna. Its accuracy, precision and recovery are well within the criteria [19–21] for biological sample analysis. We believe that this method fulfils experimental and clinical requirements for routine determining mesna and total mesna in urine. Is noteworthy that the implementation of urine analysis for mesna with the use of our method is facilitated because equipment for HPLC-UV analysis is often a part of the existing, standard instrumentation in hospital laboratories and staff is usually well experienced in its use. The UV detector is known for its stability and low demand in terms of maintenance. In the commonly used HPLC methods with electrochemical detection, although derivatization free, the accuracy and the precision of the mesna assay depend on careful maintenance to avoid contamination of the flow cell and deterioration of the electrode. The results for monitoring mesna and total mesna in urine of patients will be presented elsewhere.

Acknowledgement

The authors wish to thank the University of Lodz for financial support of this research in the form of grant no. 505/666.

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