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Facile and sensitive method for the determination of mesna in plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

The use of 2-chloro-1-methylquinolinium tetrafluoroborate, an ultraviolet tagging reagent, for the ion-pair, reversed-phase high-performance liquid chromatography of mesna in human plasma is reported. In order to achieve this objective optimization of the two-step procedure, derivatization and separation of mesna *S*-quinolinium derivative from that of other thiols present in plasma and internal standard, was investigated. The derivatization was optimized in terms of pH, reagent excess and time of the reaction, and the mobile phase in terms of ion-pairing reagent concentration, pH, organic modifier content and temperature. Baseline separation was achieved on an analytical Waters Nova-Pak C_{18} (150×3.9 mm, 5 μm) column with a mobile phase consisting of pH 2.3 0.05 *M* trichloroacetic acid–acetonitrile (89:11, v/v) pumped at 1.2 ml/min. The peak height ratios of the mesna derivative to that of the internal standard (thiomalic acid) varied linearly with the concentration of the analyte added to normal plasma with a correlation coefficient of 0.9997. The lower limits of detection and quantitation were 40 pmol/ml (0.8 pmol on-column) and 160 pmol/ml (3.2 pmol on-column), respectively. The intra-run imprecision and inaccuracy were from 1.3 to 2.4 and from 1.3 to 2.0,%, respectively.

Keywords: Derivatization, LC; Mesna; 2-Mercaptoethanesulfonic acid; Thiols; 2-Chloro-1-methyloquinolinium tetrafluoroborate; Thiomalic acid

1. Introduction

Mesna, sodium 2-mercaptoethanesulfonate, is a thiol compound highly effective in the prevention of the urinary toxicity associated with high doses of oxazaphosphorines, an alkylating cytostatics, such as cyclophosphamide and iphosphamide, frequently prescribed in the treatment of cancer or as part of the immunosuppressive conditioning regiments in bone marrow recipients [1]. The protective capability of mesna to the bladder mucosa is ascribed to the chemical reaction of the thiol group with the urotoxic acrolein and other metabolites of alkylating cytostatics [2]. Mesna does not block the antitumor action of oxazophosphorines due to its rapid formation of the inactive dimmer dimesna in the bloodstream [3]. The active monomer is formed back from dimesna in the renal tubule cells, thereby binding the toxins harmful to the genitourinary tract. The rest of

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mesna, detectable in the blood through a prolonged period of time, is potentially able to react with the 4-hydroxy metabolite of oxaphosphorines [4]. It is of great importance for understanding of the pharmacology of mesna to realize that the reaction occurs exclusively at the hydroxy group in the 4-position not at the chloroethyl moiety responsible for cytotoxic activity. To understand better the pharmacology of mesna simple and reliable analytical methods are needed. Monitoring of body fluid levels of free mesna is above all clinically important since in order to ensure constant protection of the bladder and urinary tract, sufficient levels of free thiol must be maintained during the period when the toxic metabolites are excreted.

Some time ago mesna was measured by the non-specific Ellman's free thiol assay [5], and some of its modifications [6]. However, the background concentration of endogenous and exogenous free thiols in plasma and urine is high and variable. In addition, administration of mesna markedly derange the sulfhydryl and disulfide homeostasis of physiological liquids [7], which further complicate the picture. To overcome this problem, several high-performance liquid chromatography (HPLC) procedures with ultraviolet, fluorogenic or electrochemical detection have been reported [1,8–11]. A HPLC method using ultraviolet detection at 220 nm [8] without derivatization shows very low sensitivity, and post-column derivatization approach with Ellman's reagent [10] requires special equipment which may not be readily available. Fluorogenic pre-column derivatization [11] with aroylacrylic acids was so far applied only to pharmaceutical preparations. The most popular method, electrochemical detection [1,9,10], requires careful maintenance of the flow-cell, and since a number of chemical groups in clinical samples give an electrochemical response, interference from drugs and their metabolites during multiple drug treatment could be a problem.

In this report we describe a simple and sensitive HPLC method for analysis of mesna with pre-column derivatization and ultraviolet detection. The assay uses 2-chloro-1-methylquinolinium tetrafluoroborate as a derivatization reagent and trichloroacetic acid as both an ion-pairing reagent and main mobile phase buffer component.

2. Experimental

2.1. Chemicals and solutions

2.1.1. Chemicals

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory as described in our previous report [12]. 2-Mercaptoethanesulfonic acid, sodium salt (mesna, MES) and 3-mercaptopropanesulfonic acid, sodium salt (MPS) were purchased from Aldrich Europe (Beerse, Belgium). Thiomalic acid (TMA), tri-*n*-butylphosphine (TNBP), 2-mercaptopropionic acid (2MPA), 3-mercaptopropionic acid (3MPA) and thioglicolic acid (TGA) were from Fluka (Buchs Switzerland), ethylenediaminetetraacetic acid, disodium salt (EDTA), perchloric acid (PCA), and 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.

2.1.2. Solutions

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 m*M*) were prepared in water or dilute hydrochloric acid, standardized with *o*-hydroxymercurybenzoate [13] and kept at 4°C. The working solutions were prepared daily as needed.

2.2. Instrumentation

HPLC analyses were performed with a Hewlett-Packard 1100 Series system equipped with a quaternary pump, an autosampler, a thermostated column compartment, a vacuum degasser and a diode-array detector. For instrument control, data acquisition and data analysis a HP ChemStation for LC 3D system including single instrument HP ChemStation software and a Vectra color computer was used. For pH

measurement, a Hach One pH meter was used. Water was purified using a Millipore Milli-Q RG system.

2.3. Chromatography

Samples (20 μl) were injected using an autosampler into a Waters Nova-Pak C_{18} (150×3.9 mm) column packed with 5 μm particles. Separation was isocratic using a mobile phase consisting of pH 2.3 0.05 *M* TCA–acetonitrile (89:11, v/v) pumped at 1.2 ml/min and at 40°C. The absorbances were measured at 350 nm. Identification of peaks was based on comparison of retention times and diode-array spectra with the corresponding set of data obtained by analyzing authentic compounds.

2.4. Calibration standards

For preparation of calibration standards of human plasma, 250-μl portions of mesna-free plasma from an apparently healthy donor were placed in polypropylene tubes, spiked with an appropriate amount of working standard solution of mesna and assayed according to Section 2.6.

2.5. Search for internal standard

Several thiol compounds, such as 2- and 3-mercaptopropionic acid, thioglicolic acid, thiomalic acid and 3-mercaptopropanesulfonic acid were added first to mesna water standard and next to plasma spiked with mesna and the resulted mixtures were subjected to derivatization and separation under various conditions.

2.6. Assay of mesna in plasma samples

To 250 μl of plasma, 250 μl of 0.1 *M* EDTA solution, 250 μl of 1 *M* Tris buffer solution, pH 7.4, 75 μl 1 μmol/ml of TMA (internal standard) solution and 30 μl of CMQT solution were added. After vortex-mixing the reaction mixture was put aside for 1 min, deproteinized with 150 μl of 3 *M* PCA solution and centrifuged for 10 min at 12 000 *g* followed by injection of 20 μl of the supernatant into the chromatographic system.

2.7. Method validation procedures

2.7.1. Linearity

Linearity of the assay was demonstrated by processing plasma mesna standards in triplicate at nine separate concentrations over the range of 0.16–30 nmol/ml. The peak heights or peak areas ratios of mesna derivative to that of internal standard were plotted versus analyte concentration and the curve was fitted by least-square linear regression analysis.

2.7.2. Lower limits of detection and quantitation

The limits of detection were assessed as the minimum detectable quantity 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 of quantity of which, both, inaccuracy and imprecision are within 20% [14].

2.7.3. 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 along with the internal standard were added to normal mesna-free plasma and the samples were processed according to the recommended 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 appropriate calibration curve obtained on that occasion. Imprecision was expressed in terms of the relative standard deviation (RSD), 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 was calculated with the use of formula:

$$\text{Recovery (\%)} = \frac{\text{measured amount}}{\text{added amount}} \cdot 100\%$$

3. Results and discussion

3.1. Method development

3.1.1. Derivatization

The derivatization reaction of mesna with CMQT as well as the chemical formula of TMA applied as an internal standard are shown in Fig. 1. The derivatization reaction yield was optimized in terms of pH, reagent excess and time. Kinetic studies revealed that at pH 7.4 and with eightfold reagent excess reaction is virtually instantaneous with respect to both analyte and internal standard (data not shown).

3.1.2. Spectral characterization of derivatives

The analyte and internal standard derivatives showed well defined and intensive absorption maxima, at approximately 350 nm, bathochromically shifted (Fig. 2) from the derivatization reagent maximum at 320 nm. This phenomenon is advantageous since at a detector analytical wavelength of 350 nm, absorbance of even a large excess of the reagent is relatively low, and does not complicate the chromatogram.

3.1.3. Optimal separation conditions

Each component and parameter of the mobile phase was adjusted independently to achieve optimal separation of the mesna derivative from that of internal standard and other plasma endogenous thiols such as cysteine, which are known to react with CMQT [12]. Accordingly, we have studied the influence of TCA buffer concentration, organic modifier content, pH and temperature of the mobile

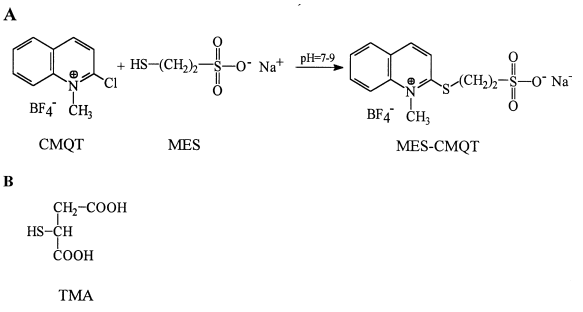

Fig. 1. Chemical derivatization reaction of MES with CMQT (A), and chemical formula of the internal standard (B).

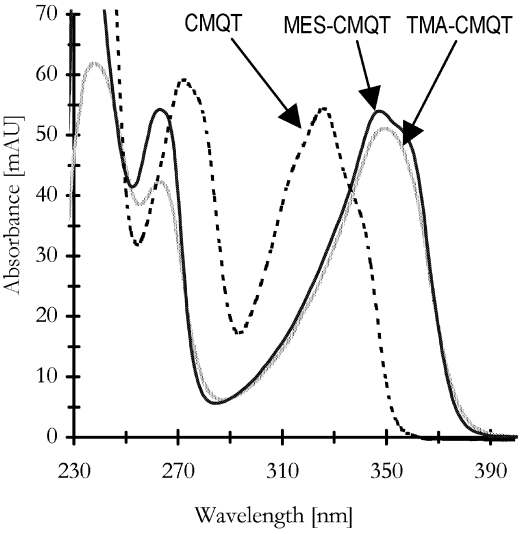

Fig. 2. Comparison of the absorption spectra of derivatization reagent and analyte as well as the internal standard.

phase on the peak height, retention factor and resolution.

The TCA buffer concentration studied ranged from 0.01 to 0.1 *M*. No effect of the counter-ion concentration on peak height of analyte (MES-CMQT) and internal standard (TMA-CMQT) derivatives was observed (data not shown). Resolution between these two peaks varied with TCA concentration passing through two maxima at 0.05 and 0.08 *M*. The capacity factor for MES-CMQT slightly increased with concentration while TMA-CMQT showed the same tendency as resolution between these two peaks (data not shown).

The dependence of quality of HPLC analysis on pH of the mobile phase was studied with a mobile phase consisting of pH 2.3 0.05 *M* TCA solution–acetonitrile (89:11, v/v) pumped at 1.2 ml/min. In the pH range investigated, from 2.2 to 4 no effect of pH on capacity factor of MES-CMQT was observed (Fig. 3A). The sulfonic acid group of the mesna moiety of MES-CMQT has a pK_a of approximately 2 [15] and is completely ionized within the pH range studied. The derivative as a whole is zwitterionic as a result of the permanent positive charge on quaternary nitrogen atom in quinolinium fragment. The capacity factor of TMA-CMQT decreased signifi-

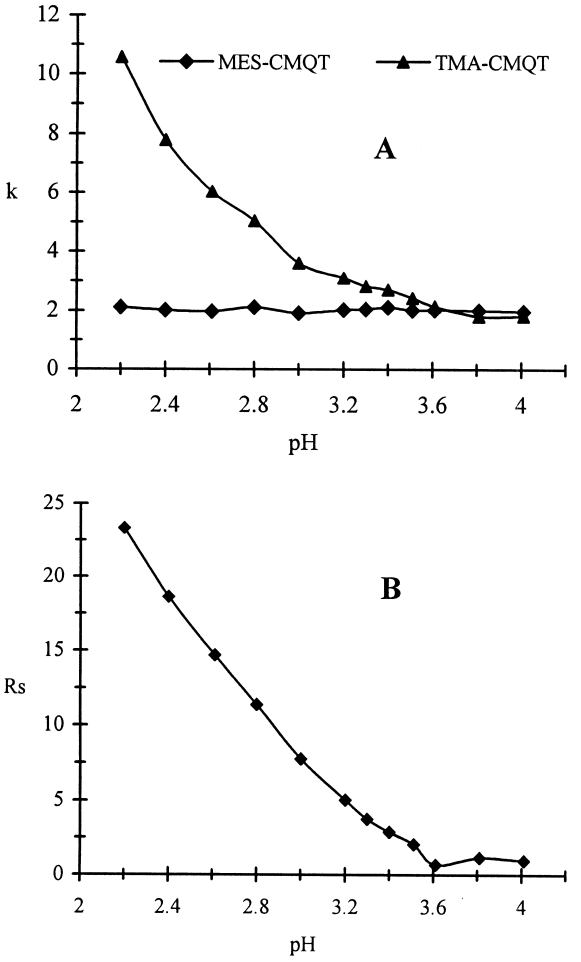

Fig. 3. Effect of the mobile phase pH on (A) capacity factors of MES-CMQT and TMA-CMQT and (B) resolution between the above mentioned derivatives.

cantly when pH was increased causing alteration of elution sequence of the two peaks, starting from pH 3.6. Growing negative charge on thiomalic moiety, resulted from progressive deprotonation of its carboxylic groups when pH increases, decreased the net positive charge of the TMA-CMQT derivative as a whole. Smaller positive charge caused weaker interaction with the trichloroacetate pairing agent and resulted in poorer retention. Resolution between MES-CMQT and TMA-CMQT decreased when pH was increased, approaching zero at pH 3.6 (Fig. 3B).

The effect of increasing the content of acetonitrile and methanol in the mobile phase was evaluated. As expected the organic modifier increase will in general be associated with a decrease in k factor and increase in peak heights. A temperature increase causes the peak height increase and decrease in k factor (data not shown).

After thorough study of the above chromatographic variables, the optimal separation conditions for plasma sample were chosen and Fig. 4 shows the final result of the procedure. The plasma matrix does not interfere with the resolution and quantitation of the resulting mesna and internal standard derivatives. The mesna and internal standard derivative elute after 1.3 and 2.5 min, respectively. Endogenous thiol derivatives and those of commonly used thiol drugs elute later.

3.2. Method validation results

3.2.1. Linearity

The relationship between detector response and mesna concentration was continuous and reproducible and was demonstrated using a nine-point calibration curve. The calibration curve was linear in the range 0.16 to 30 nmol/ml. This calibration range can be easily extended up if required. The equations for the linear regression line and coefficient of correlation were $y=0.1062x-0.0061$ and $r^2=0.9997$, respectively. The corresponding set of data using peak

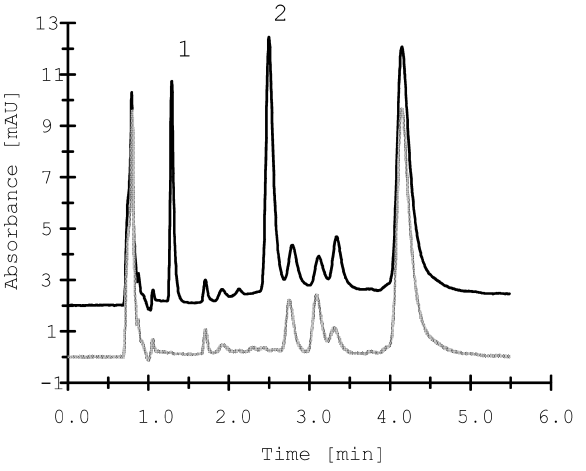

Fig. 4. HPLC chromatograms of plasma sample: thin line, blank test; thick line, spiked with mesna (8 nmol/ml, 160 pmol on-column) and treated with CMQT. Peaks: 1=MES-CMQT; 2=TMA-CMQT.

area ratios was $y = 0.0546x - 0.0230$ and $r^2 = 0.9987$, respectively.

3.2.2. Inaccuracy, imprecision and recovery

Intra-run imprecision and inaccuracy for the assay of mesna as 2-*S*-quinolinium derivative – expressed in RSD and mean relative error, respectively – were determined (Table 1) for four concentrations representing the whole range of the calibration curve. With no outliers excluded, the imprecision and inaccuracy were for mesna within 1.3–2.4 and 1.3–2.0%, respectively; and for thiomalic acid within 1.4–2.2 and 0.3–1.9%, respectively. Recovery values were for both compounds from 98.1 to 101.3%.

3.2.3. Lower limits of detection and quantitation

The lower limit of detection of mesna in human plasma was 40 pmol/ml (0.8 pmol on-column). At this concentration the signal-to-noise ratio was 3. At 160 pmol/ml (3.2 pmol on-column) the percent deviation from the nominal concentration and the RSD were both less than 2.4%, and this level of concentration was recognized as the lower limit of quantitation.

3.2.4. Internal standard

In order to correct for percent recovery, matrix effects, injection variation and column aging effects an internal standard mode of quantitation was applied. From amongst the thiol candidate internal standards tested, such as 2- and 3-mercaptopropionic acid, thioglicolic acid, thiomalic acid and 3-mercaptopropanesulfonic acid, thiomalic acid was chosen. The closest homologue of mesna – 3-mercaptopropanesulfonic acid – performed very well in the mesna water standard solution, but in the plasma samples coeluted with some of the matrix peak. *N*-Acetylcysteine, *N*-(2-mercaptopropionyl)glycine and penicillamine were not taken into consideration because they can be present in plasma as drugs.

3.2.5. Stability of the derivatives

The MES-CMQT and TMA-CMQT derivatives acidified after derivatization (Section 2.6) were monitored at ambient temperature for 2 days and no significant changes in peak heights were noticed (data not shown here).

4. Conclusion

Our results indicate that the CMQT-HPLC method of analyzing mesna is fast, sensitive and reliable. Analytical figures of merit demonstrated during the method validation procedure compare well with those of known methods for determining mesna in physiological fluids. The sensitivity owing to the high molar absorption of the mesna 2-*S*-quinolinium derivative ($\epsilon = 20\,100$ l/mol cm) is even higher than with methods using electrochemical detection known for inherent sensitivity although more demanding in terms of detector maintenance. The lower detection (LLOD) and quantitation (LLOQ) limits with the present method are lower (LLOD, 40 pmol/ml; LLOQ, 160 pmol/ml) than with electrochemical detection [1,9]. Recovery is about the same as shown by El-Yazigi et al. [1], and much higher (98.6 against 76.8%) than that reported by James and Rogers [9]. Attained imprecision (2.4%) is also better than that of the above mentioned researchers (3.5 and 6.0%, respectively).

The proposed CMQT-HPLC method can be easily adopted for analysis of free and oxidized mesna in any physiological fluid.

Table 1
Recovery, imprecision and inaccuracy for mesna and thiomalic acid analysis in the form of their 2-*S*-quinolinium derivative ($n = 4$)[a]

Nominal concentration (nmol/ml)	Recovery (%)		Imprecision, RSD (%)		Inaccuracy, E_{rel} (%)	
	MES	TMA	MES	TMA	MES	TMA
0.16	98.1	99.2	2.3	2.2	−1.7	−1.4
0.50	98.2	98.4	2.0	2.1	−2.0	−1.9
20.0	98.6	100.8	2.4	1.8	−1.4	1.1
30.0	101.3	99.7	1.3	1.4	1.3	−0.3

[a] Calculated from the peak heights.

Acknowledgements

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