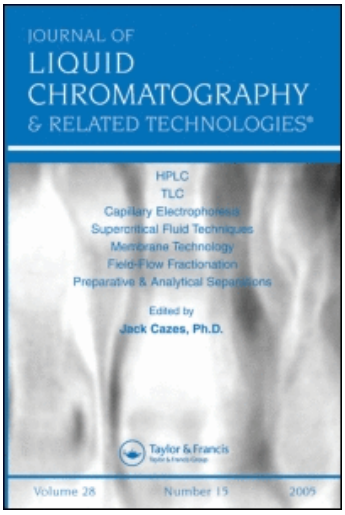

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 31 May 2001

To cite this Article Bald, Edward and Glowacki, Rafal(2001) '2-CHLORO-1-METHYLQUINOLINIUM TETRAFLUOROBORATE AS AN EFFECTIVE AND THIOL SPECIFIC UV-TAGGING REAGENT FOR LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 24: 9, 1323 — 1339
To link to this Article: DOI: 10.1081/JLC-100103450
URL: http://dx.doi.org/10.1081/JLC-100103450

J. LIQ. CHROM. & REL. TECHNOL., 24(9), 1323–1339 (2001)

2-CHLORO-1-METHYLQUINOLINIUM TETRAFLUOROBORATE AS AN EFFECTIVE AND THIOL SPECIFIC UV-TAGGING REAGENT FOR LIQUID CHROMATOGRAPHY

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ABSTRACT

A new, highly reactive, and thiol specific derivatization reagent for liquid chromatographic analysis has been developed. The reagent, 2-chloro-1-methylquinolinium tetrafluoroborate, reacts instantaneously with hydrophilic thiols in water under mild conditions. The 2-S-quinolinium derivatives, resulting from this reaction, are stable thioethers exhibiting well defined absorption maximum at 348 nm and molar absorptivity coefficient about 2 x 10^4 L mol $^{-1}$ cm^{-1}. It is shown that under reversed-phase high performance liquid chromatography conditions with gradient elution, six thiols possessing different functional groups, can be baseline separated and quantified within 4 min in one analytical run.

The lower limits of detection and quantitation of the analytes (20 μL injection volume) are within 0.3-1.5 pmol and 0.5-4.0

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pmol, respectively. The assays are linear in the range of 0.025-8 nmol/mL with correlation coefficients values close to 0.9999.

Synthesis of 2-chloro-1-methylquinolinium tetrafluoroborate, as well as isolation of the 2-S-quinolinium derivative of one model thiol, are described.

INTRODUCTION

Thiols play a specific role among reduced sulfur compounds; they are chemically and biochemically very active components of the sulfur cycle of the natural environment and have been extensively studied in various biological systems. Many biological phenomena, among others, redox-, methyl transfer-, and carbon dioxide-fixation reactions, are believed to be dependent on the presence of a thiol group. The determination of thiol containing compounds is important for biochemical research, in pharmacodynamic studies of the thiol drugs, or in the diagnosis of several diseases, e.g. cystinuria and homocystinuria. The homocysteine assay is a sensitive tool for early diagnosis of disturbed remetylation and transsulfuration leading to hyperhomocysteinemia, which in turn is associated with an increased risk of atherothrombotic vascular events.[1]

The analysis of thiols can be quite perplexing. Aside from the great susceptibility to oxidation, which can occur before or during analytical process, most thiols lack the structural properties necessary for the production of signals compatible with common HPLC detectors, such as UV absorbance and fluorescence. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulfhydryl group blocking if fluorescence or UV-VIS detection methods are employed. Numerous reagents are available for the derivatization via-SH group and subsequent HPLC analysis. A majority of the reagents can be classified by type of the reactive moiety into three categories: activated halogen compounds,[2-10] disulfides,[11-13] and compounds possessing maleimide moiety,[14-18] and are reviewed with some experimental details in some excellent work.[19,29] However, increased demand for measuring thiols, mostly biological ones in clinical practice, raises the issue of developing new methods better suited to accommodate high testing volumes and faster turnaround time.

In this report, we describe 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), which performs very well as thiol specific derivatization reagent in terms of derivatization reaction velocity, stability, chromatographic properties of the derivatives, and thus, amenability to automatization. Before introducing of this reagent, thiols were measured in our laboratory in the form of their S-pyridinium derivatives. The methodology involved chemical derivatization of the -SH group with the use of 2-halopirydinium salts to stable thioether - S-pyridinium derivative - followed by ion-pair reversed-phase HPLC separation and

spectrophotometric detection at 312 nm. Although, this method has proven to be useful in quantitative HPLC analysis of endogenous[21,22] and exogenous[4] low-molecular-weigh biological thiols in human body fluids, we have sought the development of an alternative method that takes advantage of very high reactivity of CMQT and profitable spectrophotometric and chromatographic properties of S-quinolinium derivatives.

EXPERIMENTAL

Chemicals and Solutions

Derivatization reagent (CMQT) was prepared in this laboratory. Trimethyloxonium tetrafluoroborate, N-(2-mercaptopropionyl)glycine (MPG), thiomalic acid (TMA), and 3-mercaptopropionic acid (3MPA) were provided by Fluka (Buchs, Switzerland). 2-Mercaptoethane sulfonic acid, sodium salt (mesna, 2MES) and 3-mercaptopropane sulfonic acid, sodium salt (3MPS) was purchased from Aldrich Europe (Beerse, Belgium). 2-Chloroquinoline was from Lancaster (Eastgate, England), glutathione (GSH) from Reanal (Budapest, Hungary), and trichloroacetic acid (TCA) from Merck (Darmstadt, Germany). All other chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

Stock standard solutions (10 mM) of the thiol compounds were prepared in water or dilute hydrochloric acid, kept at 4°C, and standardised with o-hydroxymercurybenzoate.[23] Working standard solutions were prepared daily by dilution with water containing 1 mmol/L of EDTA. The pH of TCA and Tris buffers was adjusted by potentiometric titrations with lithium hydroxide and hydrochloric acid, respectively. The titration system was calibrated with standard pH solutions.

Preparation of the Derivatization Reagent

To prepare 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) 2-chloroquinoline (1.000 g, 3.112 mmol), nitromethane (1.2 mL) and trimethyloxonium tetrafluoroborate (1.000 g, 6.760 mmol) were mixed together and, after evolution of dimethyl ether has ceased, diethyl ether (4 mL) was added to the reaction mixture. White precipitate was filtered off, washed with diethyl ether (2 x 2 mL), and dried over phosphorous pentoxide under vacuum, to yield 1.555 g [95.7%; m.p. 128-132°C, ^{1}H NMR, 60 MHz, D_2O, δ ppm: 9.21 (d, 1H, J = 9.0 Hz, H - 4 quin.), 8.66 - 7.23 (m, 4H, H-5-8 quin.), 6.66 (d, 1H, J=9.0 Hz, H-3 quin.), 4.91 (s, 3H, CH_3); IR, KBr, cm^{-1}: ν C-H arom. 3123-3090, ν C-H alif. 3016, δ

arom. 1618-1438) For thiol derivatization prior to HPLC, a 100 μmol/mL aqueous solution of CMQT was used.

Isolation of the 2-S-Quinolinium Derivative of Mesna

CMQT (2.150 g, 8 mmol) and 2MES (0.654 g, 4 mmol) were dissolved in 2 mL of 25% ammonium hydroxide solution and the reaction mixture was put aside for 12 h at 4°C. The reaction product, 2MES-CMQT derivative, was isolated by normal phase ion-pair chromatography with the use of sodium bromide as pairing reagent, according to a procedure described in the literature.[24] The counterion of the derivative was changed from tetrafluoroborate to bromide during purification. The structure of white higroscopic powder obtained (0.891 g, 60%, mp: 92-4°C) was characterized by spectroscopic methods. UV-VIS H_2O: λ_{max} = 348 nm, ε = 20100 L $mol^{-1}cm^{-1}$; IR (KBr), cm^{-1} : ν C-H arom. 3090, ν C-H alif. 2980, δ arom. 1641 1432, ν C-S 742; ^{1}H NMR (D_2O), 200 MHz, δ ppm: 8,59 (d, 1H, J = 9.0 Hz), 8,10-7.74 (m, 4H, H-5-8 quin.), 7.77 (d, 1H, J = 9.0 Hz, H-3 quin.), 4.30 (s, 3H, CH_3), 3.82-3.26 (m, 4H, CH_2-CH_2).

Equipment

The separation and measurement of thiol-CMQT derivatives were performed on Hewlett Packard liquid chromatograph (1100 Series system, Waldbronn, Germany) equipped with a quaternary pomp, autosampler, thermostated column compartment, vacuum degasser, and diode-array detector. For instrument control, data acquisition, and data analysis a Hewlett Packard ChemStation for LC 3D system and Vectra color computer were used. UV spectra were recorded on a Hewlett Packard HP 8453 diode-array UV-VIS spectrophotometer. Proton magnetic resonance (^{1}H-NMR) spectra were obtained on Tesla 60 BS 467 (Brno, Czech Republic) and Varian GEMINI 200 DP (Paloalto, CA, USA) spectromethers with tetramethylsilane as an internal standard. IR spectra were made with BIO-RAD FTIR 175 C spectrophotometer (Cambridge, MA, USA). Water was purified using a Millipore Milli-Q RG (Vien, Austria) system. For pH measurement, a Hach One pH-meter was used.

Derivatization Procedure

In a 5 mL calibrated flask were placed an aliquot of a thiol sample, approximately seven fold molar excess of 100 μmol/mL CMQT solution, and 500 μL of pH 8.2 1 M Tris buffer solution. The flask was then stoppered, mixed by inver-

sion, acidified with 80 µL of 2.5 M hydrochloric acid, diluted to the volume with water, and an aliquot was injected into the liquid chromatographic system. The same procedure was applied to working standard solutions of thiols to obtain a calibration graphs.

Sample Assay

A sample solution was subjected to the derivatization procedure and a 20 µL aliquot of the final analytical solution was injected into the liquid chromatograph in triplicate. The peak areas or heights were measured and the amount of each analyte of the sample was then calculated by interpolation on the calibration graph.

Chromatography

Samples (20 µL) were injected through an autosampler onto a Waters Nova-Pak C_{18}, 150 x 3.9 mm, 5 µm column. The separation of the thiol-CMQT derivatives and reagent excess was achieved using gradient elution with the mobile phase delivered at flow-rate 1.5 mL/min and 40°C. The photodiode detector was set to measure the peaks at 355 nm. The elution profile was as follows: 0-0.8 min, 11% B; 0.8-3.0 min, 11-35% B; 3.0-5.0 min, 35-11% B. Allowing an additional 0.5 min for re-equilibration with mobile phase of 11% B at the same flow rate, a injection can be repeated every 6 min. Elution solvent A was 0.05 M TCA adjusted to pH 2.3 with 0.05 M lithium hydroxide solution. Solvent B was acetonitrile. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at a real time of analysis, with the corresponding set of data obtained by analyzing authentic compounds.

Linearity, Imprecision, and Inaccuracy

Linearity of the assay was demonstrated by processing water thiol standards in triplicate at 9 separate concentrations over the range 0.025-8 nmol/mL. Peak areas or highs were plotted against thiol concentration and analysed using least-square linear regression.

Imprecision and inaccuracy were assessed in conjunction with the linearity studies on three separate occasions. Known concentrations of several thiols equivalents were added to water and processed according to recommended analytical procedure. Three concentrations were studied: one near the lower limit of quantitation, one near the centre, and one near the upper boundary of the standard

curve. Imprecision was assessed in terms of the relative standard deviation (RSD) of the measured concentrations in a replicate set, while inaccuracy was determined from the mean relative error (E_{rel}) in a replicate set, i.e. difference between measured and nominal concentrations.

Low Limits of Detection and Quantitation

The limits of detection (LOD) were assessed as the minimum detectable quantity of each compound (signal-to-noise ratio of 3:1) that could be detected without interference from the baseline noise. The limits of quantitation (LOQ) were determined as the minimum quantity of each compound at which, both, inaccuracy and imprecision is within 15%.

Stability of the 2-S-Quinolinium Derivatives

To test the stability of the 2-S-quinolinium derivatives of thiols, a solution of a mixture of 6 target analytes in water, at concentration 10 nmol/mL of each, was prepared, derivatized with CMQT according to recommended procedure, acidified with 2.5 M hydrochloric acid solution to about pH 1.5 (indicator paper), and kept at room temperature. Aliquots were injected into the chromatographic system at time zero, and in successive hours.

Effect of Acetonitrile and Mobile Phase pH on Molar Absorptivity of 2-S-Quinolinium Derivative

Isolated and purified, according to the above described procedure, a derivative of mesna in the form of bromide (2MES-CMQB) was dissolved in water to make 1.5 μmol/mL solution. A. final analytical solution of 3 nmol/mL was prepared as follows: to 10 μL of 1.5 μmol/mL of 2MES-CMQB solution was added appropriate amount of acetonitrile and 0.05 M TCA buffer of desired pH to make the volume of 5 mL. The absorbance intensity was measured at λ_{max} = 348 nm with 1 cm optical path cell and molar absorptivity coefficients were calculated.

Analysis of Mesna in Urine

A 500 μL of mesna spiked urine was introduced into a test-tube containing 25 μL of 100 nmol/mL 3MPA (internal standard), 500 μL of pH 7.2 1M Tris buffer, 250 μL of 0.1 M EDTA, and 100 μL of 10 nmol/mL CMQT solution. The

reaction mixture was vortex-mixed and put aside for at least 1 min, followed by acidification with 50 µL of 3 M perchloric acid and centrifugation at 13000 g for 10 min. A 20 µL of the supernatant was injected into the HPLC system. The measured concentration was assessed by application of an appropriate calibration curve obtained on that occasion.

RESULTS AND DISCUSSION

Derivatization Reaction

A general derivatization reaction equation of thiols with CMQT is shown in Figure 1. To test the performance of CMQT, as both chemical stabilizer of -SH group and chromophoric tag, a model compound possessing different additional functional groups were taken. These groups are: sulfonic, carboxylic, primary, and secondary amine, and peptide linkage. In all cases, the reaction was instantaneous and thiol specific. The reaction product 2-S-quinolinium derivative, a stable thioether, exhibits a well defined absorption maximum in the higher UV region. The reaction is accompanied by an analytically advantageous bathochromic shift from reagent maximum (328 nm) to the maximum of the derivative (348 nm). This phenomena is displayed in Figure 2.

The proposed derivatization scheme (Figure 1) takes advantage of great susceptibility of quinolinium molecule at 2-position to nucleophilic displacement, and the high nucleophilicity of the thiol group. We have found in this work, that CMQT was much more reactive in the nucleophilic replacement reactions with thiolate ions than 2-chloro- and 2-iodo-substituted 1-methylpyridinium salts.[22,25] This observation stands in accordance with the results of others,[26] who reported that 1-methylquinolinium iodides were 230-280 times more reactive than similar 2-substituted 1-methylpyridinium salts towards hydroxide ion nucleophile, owing to lower energies of activation and higher frequency factors.

Under the recommended derivatization conditions, formation of the 2-S-quinolinium derivatives was over within 1 min, that is virtually just after mixing of the substrates (Figure 3).

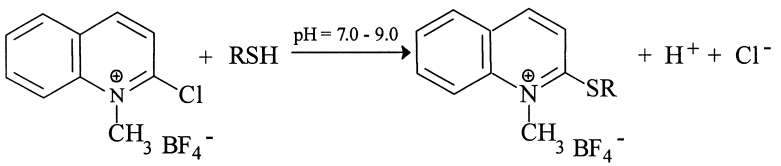

Figure 1. General chemical derivatization reaction of thiols with the use of CMQT.

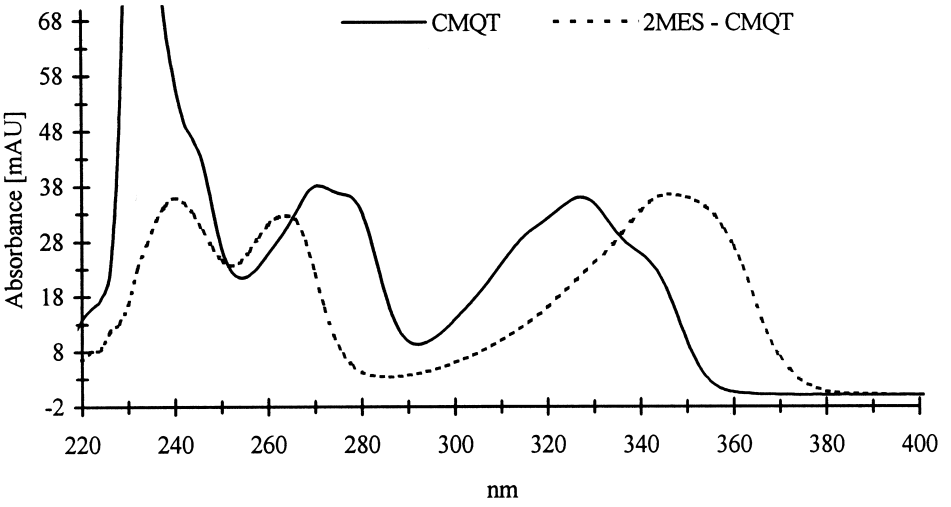

Figure 2. Comparison of absorption spectra of CMQT and 2-S-quinolinium derivative of thiol.

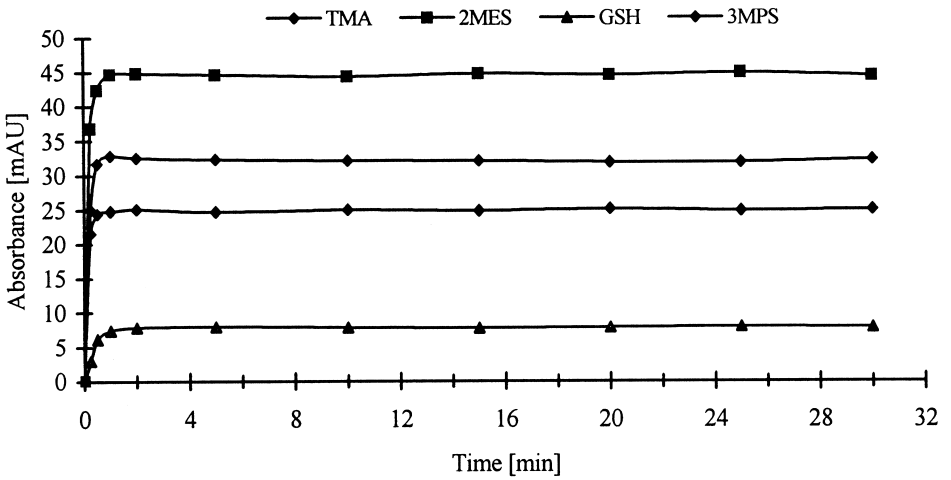

Figure 3. Kinetics of the derivatization reaction of thiols with CMQT. Conditions: pH 8.2, derivatization reagent to thiol molar ratio 7:1. Chromatographic analysis as recommended under experimental.

Chromatography

The HPLC chromatogram of the six-component mixture of thiols derivatized with CMQT is given in Figure 4. All the analytes are considered to be of interest and are baseline separated from each other and from reagent excess. The mobile phase that produced these peaks consisted of 0.05 M TCA solution adjusted to pH 2.3 with 0.05 M LiOH and acetonitrile within the range of 11-35%, added gradually according to the elution profile described under Experimental.

As can be seen from Figure 4, under these conditions all six thiol-CMQT derivatives and CMQT excess are indeed well separated within 4 min of injection. Small peaks eluting at 2.8 min constitute thiol impurity originated from commercial sample of glutathione. Its UV-spectrum taken at real time of HPLC analysis with the use of diode-array detector proved to be identical with those of other thiols. It could be γ-glutamylcysteine, a standard of which was not available in our laboratory.

TCA solution in this assay serves both as a buffer and ion-pairing reagent. This cheap, easily available mobile phase additive in reversed phase system reduces the column equilibration time to only 5-10 min. In the case of commonly used alkyl sulphonates, the equilibration takes several hours with growing tendency for increase of carbon atom number in alkyl chain.

As an organic modifier in the mobile phase, we have tried acetonitrile and methanol (data not shown). 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 time, decrease of peak highs, and higher beck pressure at an analytical column. Therefore, acetonitrile was chosen based on shorter retention time and better peak shape of analytes.

The pH of the mobile phase also affects the retention and peak shape by determining the degree of ionization. The low pH 2.3 was chosen in our chromatographic procedure in order to ensure that the functional groups of the analytes occur in a single form, whether ionized or unionized. The existence of a partially ionized analyte during a chromatographic run resulted in broad and severely tailing peaks (data not shown).

Linearity, Cross-Reactivity, and Specificity

The relationship between response and concentration of thiols was continuous and reproducible, and was demonstrated using 9-point calibration curves of each compound. The calibration curves were all linear in the range of 0.25 - 8

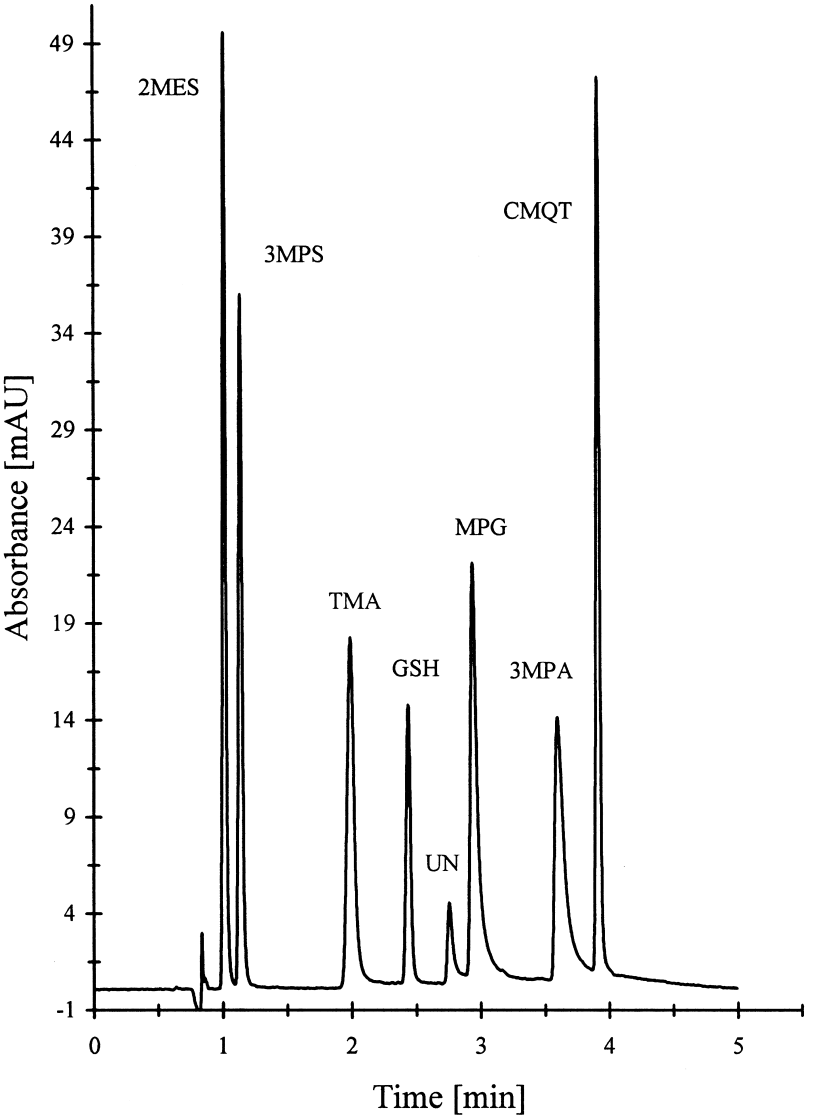

Figure 4. HPLC profile of the reaction mixture of six thiols and CMQT. 160 pmol of each thiol on column, 20 μL injection. Peaks: 2MES, 2-mercaptoethane sulfonic acid, sodium salt; 3MPS, 3-mercaptopropane sulfonic acid, sodium salt; TMA, thiomalic acid; GSH, glutathione; UN, unknown; MPG, N-(2-mercaptopropionyl)glycine; 3MPA, 3-mercaptopropionic acid; CMQT, excess of the derivatization reagents. Derivatization and chromatographic conditions as described under experimental.

nmol/mL with r^2 values close to 0.9999. This calibration range can be easily extended up if required. Detailed calibration -validation dates for the CMQT-HPLC analysis of thiols are shown in Table 1.

The calibration curves and chromatogram were not disturbed when to the thiol mixture serine was added in a large excess. It was assumed that no multiple CMQT derivatives were formed, despite the presence of another functional group ($-NH_2$, -COOH, $-SO_3H$) in the reaction mixtures potentially able to accomplish nucleophilic attack on the 2-position of the quinolinium molecule.

Imprecision, Inaccuracy, and Limits of Detection and Quantitation

Imprecision and inaccuracy for the analysis of target thiols - expressed in relative standard deviation and mean relative error, respectively - were determined (Table 2) for three concentrations of each thiol representing an entire range of the calibration curve. In all instances, imprecision and inaccuracy were within 2.4 and 4.26%, respectively, with no outliners excluded.

The lower limit of quantitation (LOQ) being the lowest concentration on the standard curves that can be measured with acceptable accuracy, precision, and variability, was deemed to be within the range of 0.5 - 4.0 pmol on the column (Table 1). A low limit of detection was assessed to be from 0.32 pmol for 2MES to 1.5 pmol on column for 3MPA (Table 1). LOQ and LOD values for the particular thiol can be extended down by arranging its favourable chromatographic conditions.

Effect of Buffer pH and Acetonitrile Content in the Mobile Phase on Absorbance Intensity

As can be seen from Figure 5, molar absorption coefficient does not change dramatically with variation of the mobile phase composition and pH value. In the range of acetonitrile, content recommended in the elution profile (11-35%)and pH 2-4 is virtually steady.

Stability of the Tiol-CMQT Derivatives

2-S-Quinolinium derivatives of the title thiols were found to be stable when kept at room temperature in water solution at pH 1.5 and concentration 10 nmol/mL for at least 25 h (Figure 6), and at pH 8.2 for at least 30 min (Figure 3). A longer time was not investigated.

Table 1. Validation Data for Determination of Thiols by CMQT - HPLC Method (n=3)

Thiol	Retention [min]	Equation	r^2	Range [nmol/ml]	RSD [%] [max]	RSD [%] [min]	Symmetry	LOQ [pmol]	LOD [pmol]
2MES	1.01	y = 6.69x + 0.32	0.9996	0.025-8.0	10.2	0.9	0.83	0.5	0.32
3MPS	1.13	y = 4.77x + 0.15	0.9997	0.025-8.0	11.0	0.7	0.86	0.5	0.46
TMA	1.99	y = 2.38x − 0.01	0.9999	0.10-8.0	12.6	0.6	0.79	2.0	0.96
GSH	2.44	y = 1.88x + 0.04	0.9998	0.05-8.0	12.4	0.7	0.88	1.0	0.70
MPG	2.94	y = 2.86x + 0.16	0.9996	0.10-8.0	11.6	2.5	0.49	2.0	0.77
3MPA	3.60	y = 1.74x − 0.17	0.9998	0.20-8.0	9.5	0.8	0.43	4.0	1.50

Table 2. Imprecision and Inacuracy for Thiol Assay in the Form of 2-S-Quinolinium Derivatives (n=5)

Thiol	Nominal Concentration [nmol/ml]	Imprecision RSD [%]	Inaccuracy E_{rel} [%]
	2.0	2.4	2.93
2MES	4.0	1.2	1.65
	8.0	0.9	−0.73
	2.0	2.0	2.08
3MPS	4.0	1.5	1.73
	8.0	0.6	−0.65
	2.0	2.4	0.54
TMA	4.0	1.0	0.71
	8.0	0.6	−0.26
	2.0	2.3	2.08
GSH	4.0	0.9	0.67
	8.0	0.7	−0.39
	2.0	1.5	4.26
MPG	4.0	1.3	1.53
	8.0	0.7	−0.68
	2.0	3.2	3.01
3MPA	4.0	2.3	−0.69
	8.0	0.8	0.41

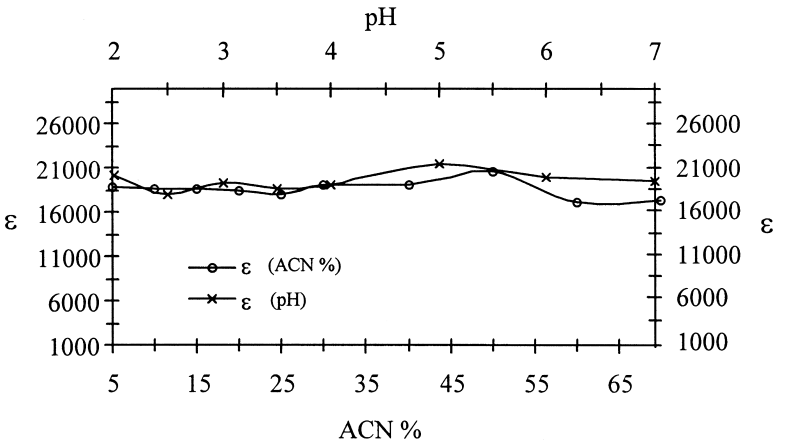

Figure 5. Absorption as a function of the buffer pH and acetonitrile content in the mobile phase. Experimental details and abbreviations as described under Experimental.

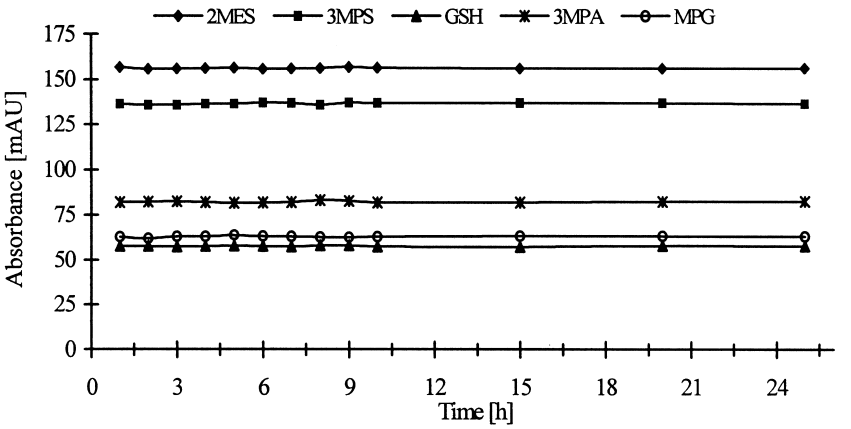

Figure 6. Stability of the 2-S-quinolinium derivatives in water solution of pH 1.5. Analytical conditions and abbreviations as described under Experimental.

Application of the Method

The method can be applied for HPLC analysis of endo- and exogenous thiols in human physiological liquids. Figure 7 shows a chromatogram of mesna, a thiol compound highly effective in the prevention of the urinary toxicity associated with high doses of oxazaphosphorines frequently prescribed in treatment of cancer. Since a sulfonic acid group of the mesna moiety of 2MES-CMQT derivative is completely ionized within the pH range permissible for a silica-based stationary phase, and consequently, the derivative as a whole is zwitterionic, the analysis of mesna in urine could be done under very simple chromatographic conditions. No buffer, ion-pairing reagent, or gradient elution were needed.

As can be seen in Figure 7, the urine matrix does not interfere with the resolution and quantitation of the mesna and internal standard 2-S-quinolinium derivatives. Sensitivity, precision, and accuracy of the method fulfils clinical and experimental requirements for determination of mesna in urine.

CONCLUSION

Analytical figures of merit, demonstrated during the method validation process, justify a conclusion that the present procedure for HPLC determination of thiols, based on precolumn derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT-HPLC), is reliable and robust. The principal feature of the utility of CMQT is simplicity of the analytical procedure without compromis-

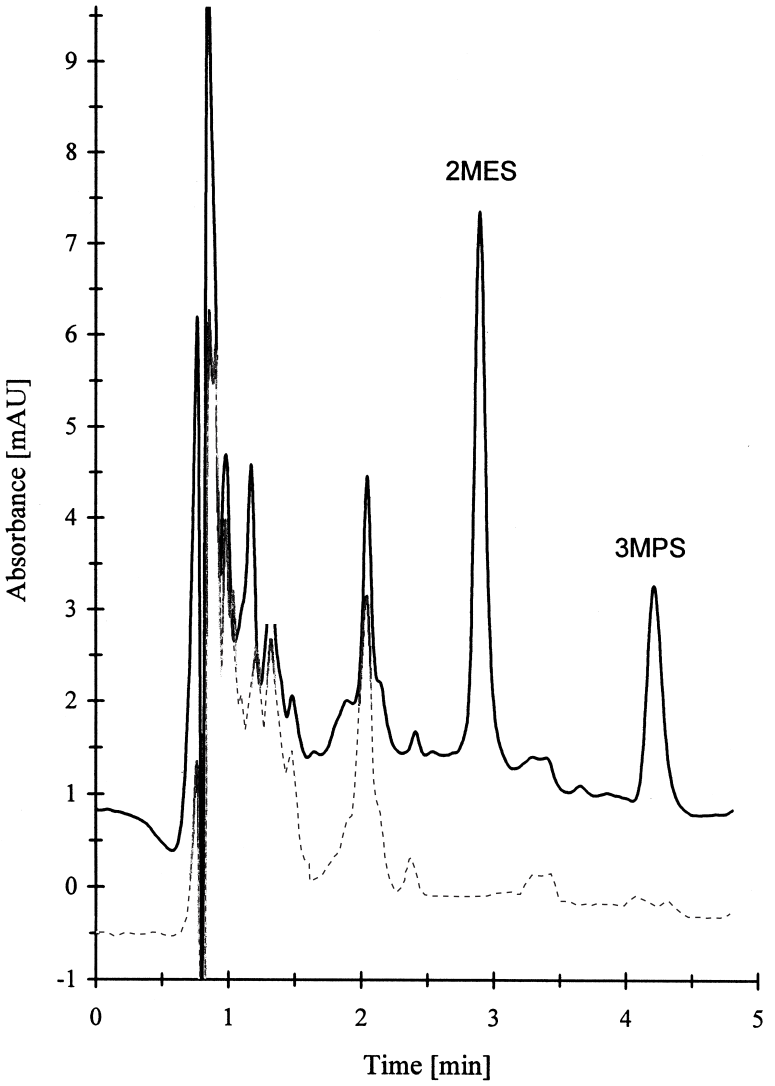

Figure 7. HPLC chromatogram of urine. Dashed line, urine blank; black line, urine spiked with mesna (2MES) solution to give concentration of 9 nmol/mL urine. Peaks: 2MES (60 pmol on column); 3MPS (internal standard, 33 pmol on column). Chromatographic conditions: Waters Nova-Pak C18 column (150 x 3.9 mm, 5 μm), isocratic elution at room temperature with a mixture of water and methanol in a ratio of 89 to 11 (v/v) pumped at 1.2 mL/min, peaks monitored at 355 nm.

ing the sensitivity. The derivatization reaction mixture is ready to be chromatographed just after mixing of the substrates, and HPLC separation takes only 4 min. In terms of sensitivity, the CMQT-HPLC method approaches the achievements of well established methods with fluorescence detection, known for their inherent sensitivity.

For example, glutathione can be detected in an ABDF-HPLC method[27] at the level of 280 fmol versus 700 fmol with the present CMQT-HPLC method. In addition, application of CMQT has several advantages: (1) high solubility and stability of CMQT and its thiol derivatives in water, (2) high optical yield of thiol derivatives at absorption maximum falling in relatively clean higher UV-region, (3) good compatibility with reversed-phase HPLC conditions and amenability to automation, and (4) chip reagents. The slightly modified CMQT-HPLC procedure in terms of chromatographic conditions is now being successfully applied to analysis of thiols in human physiological fluids by manual, as well as, fully automatic manners.

ACKNOWLEDGMENTS

The authors wish to thank Professor Bogusław Kryczka from this University for helpful discussions. This work was partly supported by Grant No 0165/T09/2000/18 from the Committee for Scientific Research and Grant No 505/268 from the University of Lodz.

REFERENCES

1. Hankey, G.J.; Eikelboom, J.W. Lancet **1999**, *354*, 407.
2. Fahey, R.C.; Newton, G.L.; Dorian, R.; Kosower, E.M. Anal. Biochem. **1981**, *114*, 383.
3. Imai, K.; Toyo'oka, T.; Watanabe, Y. Anal. Biochem. **1983**, *128*, 471.
4. Bald, E.; Sypniewski, S.; Drzewoski, J.; Stępień J. Chromatogr., B **1996**, *681*, 283.
5. Toyo'oka, T.; Suzuki, T.; Saito, Y.; Uzu, S.; Imai, K. Analyst **1989**, *114*, 413.
6. Valdez, D.; Reier, J.C. J. Liq. Chromatogr. **1987**, *10*, 2133.
7. Kawahara, Y.; Hisaoka, M.; Yamazaki, Y.; Inage, A.; Marioka, T. Chem. Pharm. Bull. **1981**, *29*, 150.
8. Lewis, P.A.; Woodward, A.J.; Maddock, J. J. Pharm. Sci. **1984**, *73*, 996.
9. Yoshida, T. J. Chromatogr., B **1996**, *678*, 157.
10. Imai, K.; Fukushima, T. Biomed. Chromatogr. **1994**, *8*, 107.
11. Jenke, D.R.; Brown, D.S. Anal. Chem. **1987**, *59*, 1509.
12. Nishiyama, J.; Kunimori, T. Anal. Biochem. **1984**, *138*, 95.

13. Andersson, A.; Isaksson, A.; Brattström, L.; Hultberg, B. Clin. Chem. **1993**, *39*, 1590.
14. Kanaoka, Y. Angew. Chem. Int. Ed. Engl. **1977**, *16*, 137.
15. Nakashima, K.; Umekawa, C.; Yoshida, H.; Nakatsuji, S.; Akiyama, S. J. Chromatogr. **1987**, *414*, 11.
16. Schreurs, M.; Hellendoorn, L.; Gooijer, C.; Velthorst, N.H. J. Chromatogr. **1991**, *552*, 625.
17. Kagedal, B.; Källberg, M. J. Chromatogr. **1982**, *229*, 409.
18. Miners, J.O.; Fearnley, I.; Smith, K.J.; Birkett, D.J.; Broaks, P.M.; Whitehouse, M.W. J. Chromatogr. **1983**, *275*, 89.
19. Shimada, K.; Mitamura, K. J. Chromatogr., B **1994**, *659*, 227.
20. Lunn, G.; Hellwig, L.C. *Handbook of Derivatization Reactions for HPLC;* John Wiley and Sons Inc.: New York,1998; 1539.
21. Kaniowska, E.; Chwatko, G.; Głowacki, R.; Kubalczyk, P.; Bald, E. J. Chromatogr., A **1998**, *798*, 27.
22. Bald, E.; Kaniowska, E.; Chwatko, G.; Głowacki, R. Talanta **2000**, *50*, 1233.
23. Wroński, Talanta **1977**, *24*, 347.
24. Bluhm, L.H.; Li, T. Tetrahedr. Lett. **1998**, *39*, 3623.
25. Sypniewski, S.; Bald, E. J. Chromatogr., A **1999**, *676*, 321.
26. Barlin, G.B.; Benbow, J.A. J.C.S. Perkin II **1975**, *298*.
27. Imai, K.; Toyo'oka, T. Methods Enzymol. **1987**, *143*, 67.

Received October 24, 2000 Manuscript 5418
Accepted January 4, 2001