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Determination by liquid chromatography of free and total cysteine in human urine in the form of its *S*-quinolinium derivative

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of free and total cysteine in urine is described. The method involves reductive conversion of cysteine dimer and cysteine mixed disulphides to their reduced counterpart with the use of tri-*n*-butylphosphine, ultraviolet-labeling with 2-chloro-1-methylquinolinium tetrafluoroborate, and liquid chromatographic separation with isocratic conditions. In developing this method the following parameters were investigated and optimized: the time, pH and reagent excess in the derivatization step, and mobile phase buffer concentration, pH, organic modifier and column temperature in the separation step. The method provides quantitative information on free and total cysteine based on assays with derivatization before and after reduction with tri-*n*-butylphosphine. The calibration graph, obtained with the use of normal urine spiked with growing amounts of cystine, was linear over the concentration range covering most experimental and clinical cases. The assay has a low pmol detection and quantitation limits, low imprecision and high recovery. The method was validated for urine samples received from several donors. Cystine was chosen as a primary calibrator for these assays.

Keywords: Derivatization, LC; Amino acids; Cysteine; Cystine; 2-Chloro-1-methylquinolinium tetrafluoroborate

1. Introduction

Cysteine, metabolically related to homocysteine and glutatione, is involved in a variety of important cellular functions, among others protein synthesis, detoxification and metabolism. Disorders of cysteine metabolism [1] include cystinosis, an autosomal recessive disease produced by a defect in lysosomal transport, and cystinuria, a common heritable disorder of the amino acids cystine, lysine, ornithine and arginine transport. The defect leads to a high concentration of these compounds in urine because their reabsorption mechanism in kidneys does not function. Due to very low solubility of cystine in urine, a kidney-stone formation is the clinical symptom of classical cystinuria. In the laboratory diagnosis of cystinuria, following positive sodium nitroprusside test, quantitative amino acids, cysteine and homocysteine, analysis is required in order to differentiate between cystinuria and homocystinuria. Altered levels of cysteine have been implicated in hyperhomocysteinemia [2,3] and in number of pathological conditions including Alzheimer's and Parkinson's disease [4] as well as autoimmune deficiency syndrome [5]. The urinary excretion of cysteine in tumour patients during ifosfamide/mesna therapy significantly increases [6] and changing sulfhydryl status of cells, due to decrease of circulating concentration of total cysteine, can thereby influence the response of the organism to the cytotoxic effects of chemotherapy. To elucidate the function of this important amino acid in biochemical and clinical

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practice its identification and determination in urine, which mirrors to some extend changes in plasma, is essential and several methods have been described in a large number of original papers and reviews [7,8].

The assay of cysteine in biological fluids is complicated by the ease with which this amino acid forms a number of different disulphides. In the case of urine cysteine forms disulphide bonds mostly with itself and with homocysteine. More importantly, cysteine, similar to majority of biologically relevant amino acids, does not show structural properties necessary for the production of signals compatible with common HPLC detectors. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulfhydryl group blocking. The most widely used fluorimetric assays have measured cysteine derivatized with monobromobimane (mBrB) [9,10], 4-(*N*,*N*-dimethyloaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBDF) [11,12] or phenylisothiocyanate [13]. An HPLC method based on acid-catalysed conversion of cysteine in biological samples to its *S*-nitroso derivatives by excess of nitrite has also been proposed [14]. Selective determination of cystine by HPLC can be accomplished by blocking cysteine with excess of suitable reagent e.g. *N*-ethylmaleimide [10,15] followed by reduction and derivatization or using dual electrochemical detection without derivatization [16]. Although each assay procedure has its advantages, the great reactivity of 2-chloro-1-methylquinolinium tetrafluoroborate under mild conditions towards sulfhydryl group and simple HPLC separation of its derivatives led us to choose it for determining cysteine.

The purpose of this study was to develop an HPLC assay method for the simple, rapid and specific analysis of free and total cysteine in human urine. The method relies on transformation of cysteine to its *S*-quinolinium derivative, separation by ion-pairing reversed-phase HPLC, and UV-absorbance detection and quantitation. Total cysteine is determined by reductive conversion of its oxidized fraction to the thiol form before the derivatization step. It was thanks to a good chromatographic properties and high optical yield of cysteine *S*-quinolinium derivative, that we could apply by far the most commonly used ultraviolet detection technique without compromising the sensitivity.

2. Experimental

2.1. Chemicals and solutions

Ethylenediaminetetraacetic acid disodium salt (EDTA), perchloric acid (PCA) and acetonitrile (MeCN) HPLC grade were purchased from J.T. Baker (Deventer, Netherlands). Tri-*n*-butylphosphine (TNBP), 2-mercaptopropionic acid (2MPA), 3-mercaptopropionic acid (3MPA), 3,3′-dithiodipropionic acid (3,3′MPA), thioglicolic acid (TGA) and thiomalic acid (TMA) were from Fluka (Buchs, Switzerland), L-cysteine (CSH) from Reanal (Budapest, Hungary), and DL-cystine (CSSC) from Lancaster (Eastgate, UK). 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was synthesized in this laboratory as described in our previous work [17]. For thiol group derivatization prior to HPLC analysis, a 100 μmol/ml water solution of CMQT was used. All other chemicals were HPLC or analytical reagent grade purchased from commercial sources. Purified water was used throughout the experiments, and all liquids used for HPLC system were filtered through 0.2 μm membranes. 0.05 *M* Trichloroacetic acid (TCA) and lithium hydroxide (LiOH) solutions were prepared by dissolving in water appropriate amounts of TCA and LiOH (8.17 g and 2.10 g, respectively) in 1 l volumetric flasks. Phosphoric buffer (0.1 *M*, pH 8.2) was prepared by mixing of 0.1 *M* potassium dihydrogen phosphate solution (1.36 g in 100 ml) and 0.1 *M* trisodium phosphate 12 hydrate solution (3.80 g in 100 ml). The pH of the buffers was adjusted by potentiometric titrations. The titration system was calibrated with standard pH solutions.

2.2. Instrumentation

Sample HPLC analysis was performed on a Hewlett-Packard (Waldbronn, Germany) HP 1100 liquid chromatograph encompassing a quaternary pump, 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 including single instrument HP ChemStation software and Vectra color computer was used. UV

spectra were recorded on a Hewlett-Packard HP 8453 diode-array UV–vis spectrophotometer. For pH measurement, a Hach One pH meter was used. Water was purified using a Millipore Milli-Q RG system.

2.3. Sample assay

2.3.1. Total cysteine, procedure 1

The assay required 500 μl of urine which was reduced with 25 μl of 10% (v/v) TNBP at 60°C for 30 min in a test tube containing the internal standard (50 μl of 1 μmol/ml 3,3′MPA), 500 μl of 0.1 *M* pH 8.2 phosphoric buffer, and 250 μl of 0.1 *M* EDTA. After incubation and cooling, 50 μl of 100 μmol/ml CMQT was added, vortex-mixed, kept at room temperature for 2 min, and followed by addition of 100 μl of 3 *M* PCA the sample was centrifuged at 13 000 *g* for 10 min before supernatant was analysed by HPLC.

2.3.2. Free cysteine, procedure 2

A 500 μl sample of urine was derivatized with 50 μl of 100 μmol/ml CMQT in a test tube containing the internal standard (50 μl of 1 μmol/ml 3MPA), 500 μl of 0.1 *M* pH 8.2 phosphoric buffer and 250 μl of 0.1 *M* EDTA. The mixture was vortex-mixed and further processed as in the case of total cysteine in procedure 1.

2.4. HPLC conditions

We injected 20 μl of the supernatant onto a 150×4.6 mm, 5 μm Zorbax Eclipse XDB-C_8 column (Hewlett-Packard). The mobile phase (flow-rate, 2 ml/min; temperature, 40°C) consisted of 0.05 *M* TCA buffer, adjusted to pH 3.25 with lithium hydroxide solution of the same concentration, and acetonitrile in the ratio of 84:16 (v/v). Identification of peaks was based on comparison of retention times and diode-array spectra, taken at real time of analysis, with the corresponding set of data obtained by analyzing authentic compounds. The detector was set to measure the peaks at 355 nm.

2.5. Calibration standards

Stock solutions of 10 μmol/ml cysteine, cystine and other disulphides needed in the method development procedure were prepared by dissolving appropriate amount of the compound in 2 ml of 0.1 *M* hydrochloric acid and diluting to the volume of 10 ml. The working solutions were prepared by dilution with water as needed. For preparation of calibration standards of human urine, portions of 500 μl of urine from healthy donors were placed each in a sample tube containing a 50 μl of 1 μmol/ml 3,3′MPA (internal standard) and spiked with the growing amount of working standard solution of cystine to provide, assuming 100% of the future reduction of the disulphide bonds, concentration of exogeneous cysteine of 40, 80, 120, 160, 200, 400, 600 and 800 nmol/ml urine.

2.6. Calibration curve

Calibration curve for urine cysteine was constructed by adding of known amounts of cystine and internal standard to normal urine followed by analysis according to the procedure 1 (see Section 2.3.1). The range of cystine added was from 20 to 400 nmol/ml urine. The peak height and peak area ratios of cysteine–CMQT derivative to that of internal standard were plotted versus analyte concentration and the curve was fitted by least-square linear regression analysis.

2.7. Search for internal standard

Several thiols, in the disulphide form, were tested as candidate internal standard. All these compounds were added to cystine water standard solution to achieve a final concentration (in respect to each) of 10 nmol/ml, and the resultant mixture was subjected to reduction, derivatization and HPLC separation under various conditions.

3. Results and discussion

The procedure that is recommended for determination of urine total cysteine encompasses three steps: (1) generation of free thiol from disulphide in order to make it accessible to the derivatization reagent; (2) derivatization in order to block the labile

thiol group and to enhance the detector signal, and (3) separation and quantitation by reversed-phase HPLC with ultraviolet detection. The protocol for the assessment of free fraction of cysteine includes steps (2) and (3) only.

3.1. Generation of free thiol

In order to determine total cysteine in urine its disulphide form must be converted to free thiol. This was done by the use of tri-*n*-butylphosphine. The data on optimal conditions for cystine disulphide cleavage with the use of TNBP were described in our earlier work [18]. Within the present work we have learned that disulphide bond of 3,3′-dithiodipropionic acid (Fig. 1B), serving as an internal standard, can be easily broken down under the same experimental conditions (data not shown).

3.2. Derivatization

Derivatization reaction equation of cysteine with CMQT is shown in Fig. 1A. CMQT reacts rapidly and specifically with the –SH group of cysteine to form stable thioether linkage, 2-*S*-quinolinium derivative, possessing a well defined absorption maximum in the upper ultraviolet region with a high molar absorptivity coefficient. The reaction occurs in a fully water environment and under mild conditions. Bathochromic shift accompanying the reaction, displayed in Fig. 2, from the reagent maximum at 328 nm to that of the derivative at 348, is analytically advantageous. It was thanks to this phenomena that we could recommend the use of a significant excess of CMQT in order to drive the reaction to the completion instantaneously (Fig. 3A and C) and avoid a huge peak of unreacted derivatization reagent on the chromatogram.

The pH effect on the derivatization yield was studied in a range between 7 and 9. As can be seen from Fig. 3B, the yield of cysteine derivative remains constant, but for reasons which we do not understand, the yield of internal standard derivative drops significantly within the pH range from 7.4 to 8.2. For routine derivatization, pH 8.2 was chosen.

3.3. Optimum separation conditions

In order to establish optimum RP-HPLC conditions for the separation of cysteine–CMQT derivative from that of internal standard, reagent excess and other matrix components several mobile phase compositions, as well as, different flow-rates and temperatures were tested.

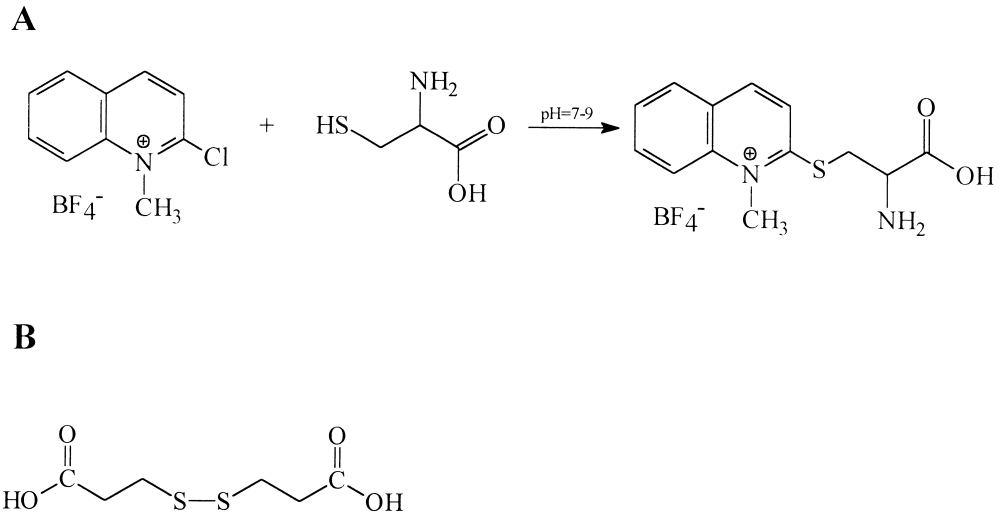

Fig. 1. Derivatization reaction equation of cysteine with 2-chloro-1-methylquinolinium tetrafluoroborate (A); Chemical structure of the internal standard (B).

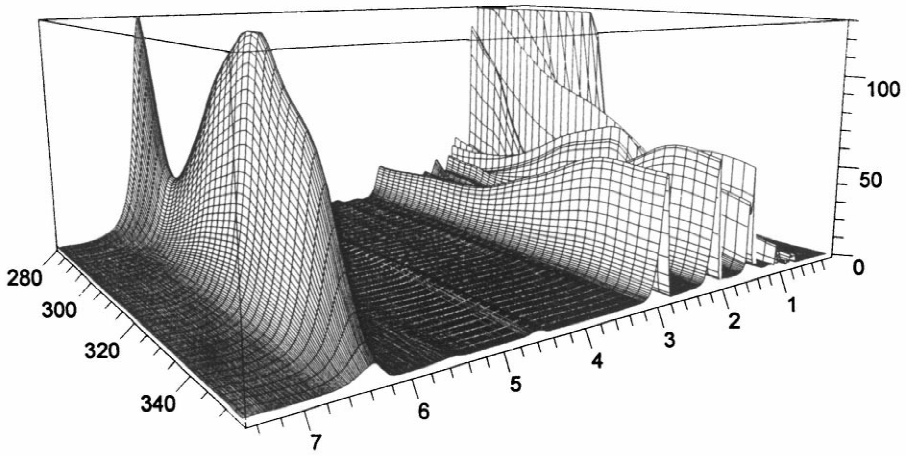

Fig. 2. Three-dimensional chromatogram of a urine sample, made with continuous spectral scanning during the elution, after successive treatment with TNBP and CMQT. Peaks from left; CMQT excess, CSH derivative and 3MPA (internal standard) derivative. Axis titles: *x*, time; *y*, wavelength and *z*, absorbance.

3.3.1. Effect of TCA concentration

Effect of TCA mobile phase buffer concentration on peak heights, retention factors and resolutions is demonstrated in Fig. 4. Retention factors and resolutions increase when buffer concentration increase whilst peak heights decrease.

3.3.2. Effect of TCA buffer pH

The pH effect was studied in a range between 3 and 4 which was imposed by good TCA buffer capacity from one side and low pH resistance of the Zorbax Eclypse C_8 from the other. As can be seen from Fig. 5, retention factors of target solutes, 3MPA peak height and resolution between 3MPA and unknown significantly decrease throughout the range. Resolution between 3MPA and CSH nearly remains steady.

3.3.3. Effect of organic modifier

As expected, an increase in acetonitrile content in the mobile phase produced decrease in all *k* values. Resolution data mirrors the same tendency. The peak heights of both, cysteine and internal standard, were positively correlated to the acetonitrile share in the mobile phase, reaching maximum at 23% and then dropped significantly. (Fig. 6).

3.3.4. Effect of flow-rate and temperature

The influence of temperature and flow-rate of the mobile phase on separation quality was also investigated (data not shown here). An increase in the column temperature caused a linear decrease of all *k* and R_s values. The peak heights showed positive correlation to the temperature increase until 45°C and then decreased firmly. The flow-rate did not have significant influence on the results.

Taking under consideration the results of the above, briefly-mentioned studies, we have chosen chromatographic conditions under which cysteine CMQT derivative eluted after 2.94 min (±SD 0.002; RSD 0.07%; $n=6$). Typical chromatograms for human urine treated and untreated with reducing agent and derivatized with CMQT can be seen in Fig. 7. The peak of CSH–CMQT derivative was apparently well separated from other matrix components including internal standard (3MPA–CMQT), eluting after 2.13 min (±SD 0.009; RSD 0.45%; $n=6$), excess of derivatization reagent (CMQT, 6.4 min) and other endogeneous urine thiols, such as homocysteine, which are known to react with CMQT and elute much earlier, close to the solvent front [17]. Commonly used thiol drugs, such as *N*-acetylcysteine, tiopronin, mesna, behave in the similar manner and do not complicate the chromatogram.

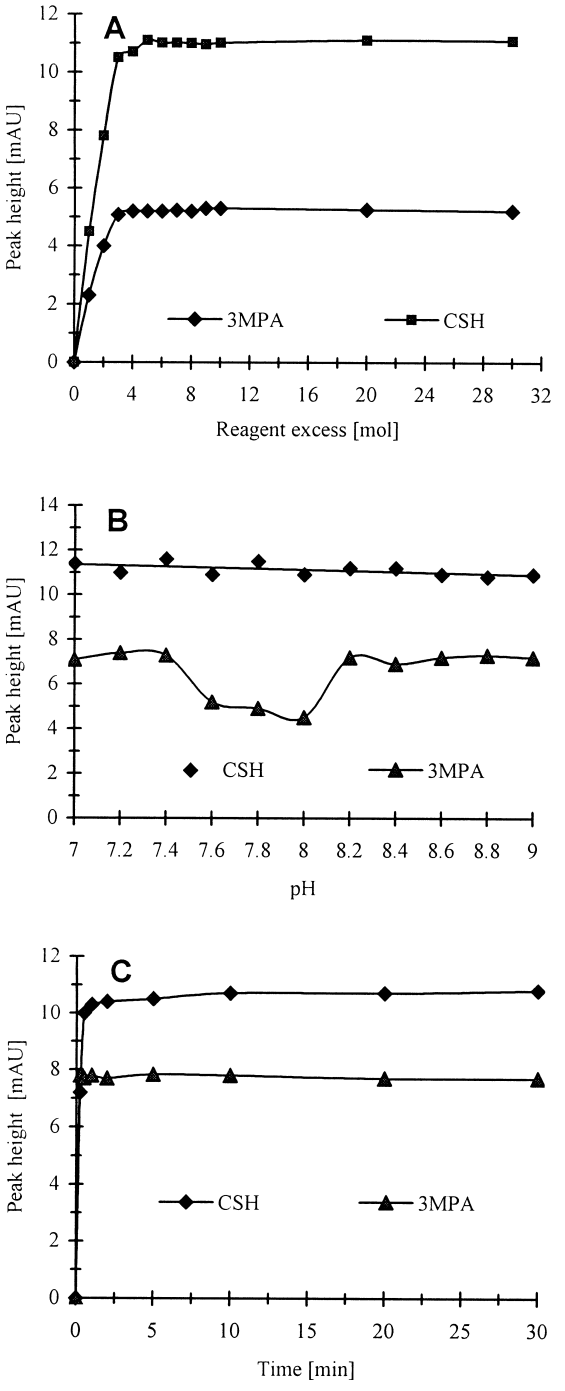

Fig. 3. Derivatization reaction yield as a function of: (A), the reagent excess; (B), the pH; (C), the time. Other conditions: (A), pH 8.3, time 3 min; (B), five-fold reagent excess, time 3 min; (C), pH 8.3, five-fold reagent excess.

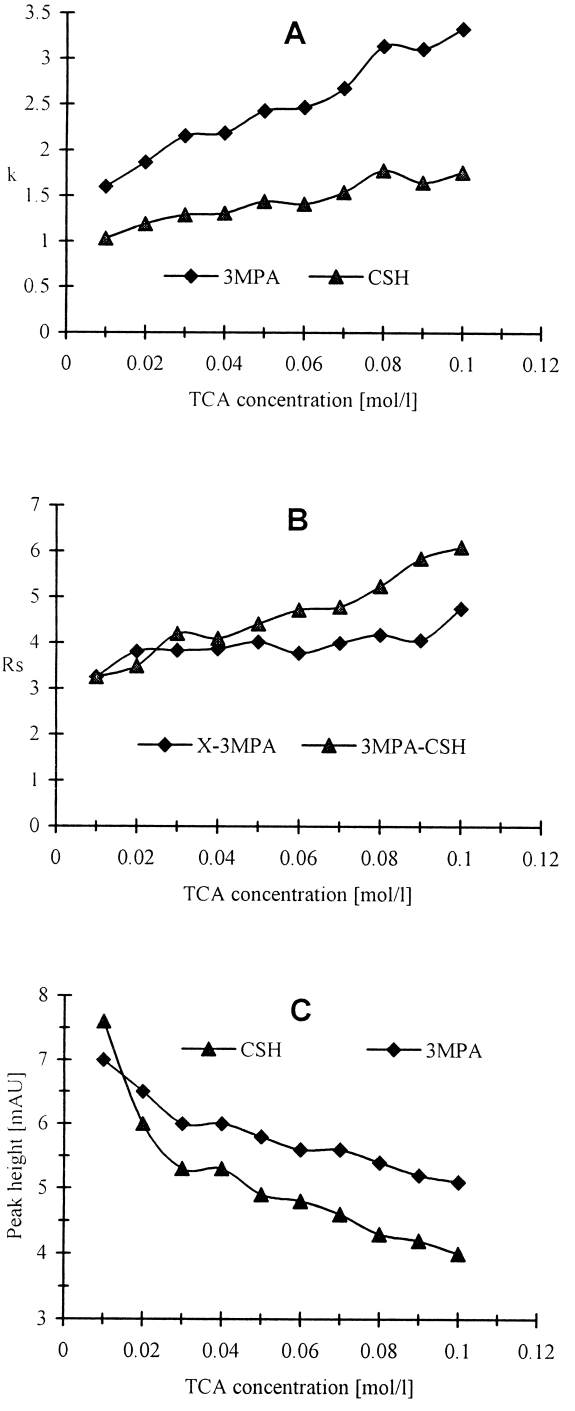

Fig. 4. Effect of the TCA buffer concentration on: (A), retention factors; (B), resolution; (C), peak heights. Other chromatographic conditions as described in Section 2.4.

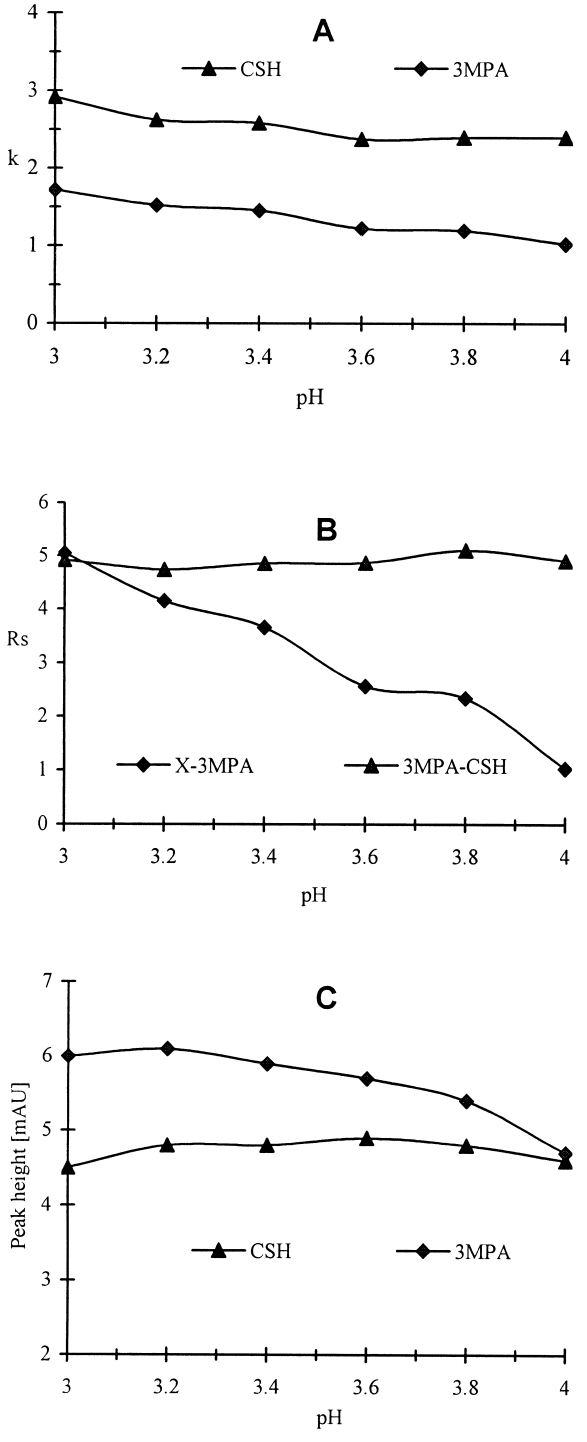

Fig. 5. Effect of the TCA buffer pH on: (A), retention factors; (B), resolution; (C), peak heights. Other chromatographic conditions as described in Section 2.4.

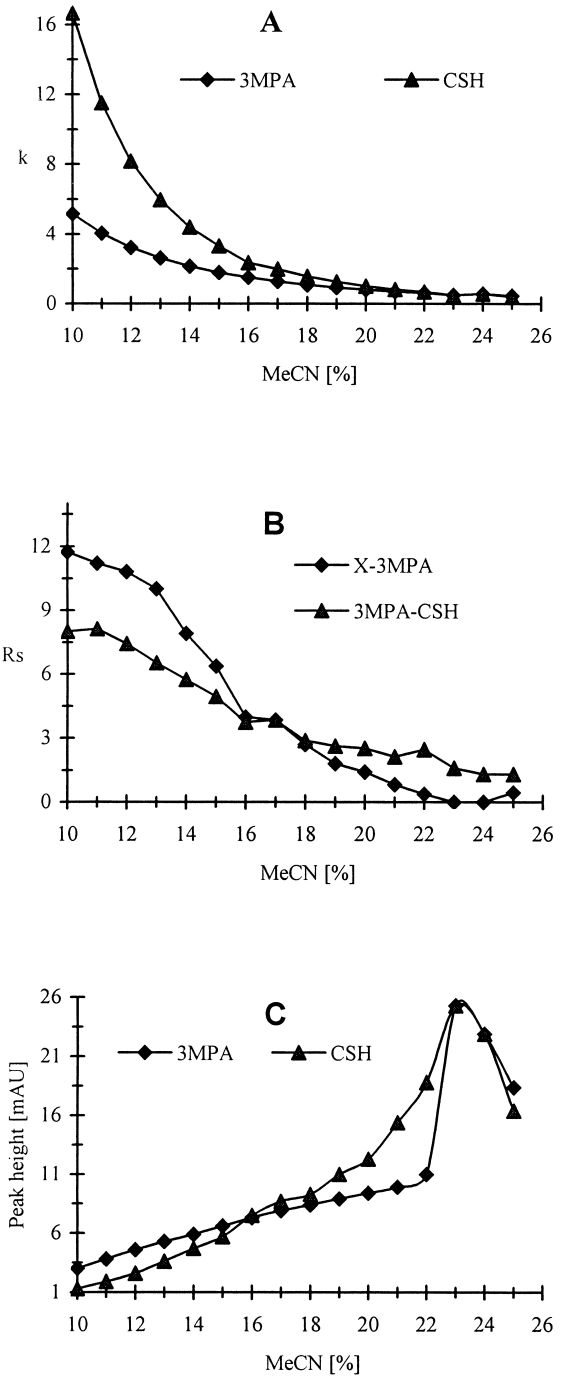

Fig. 6. Effect of the mobile phase acetonitrile content on: (A), retention factors; (B), resolution; C, peak heights. Other chromatographic conditions as described in Section 2.4.

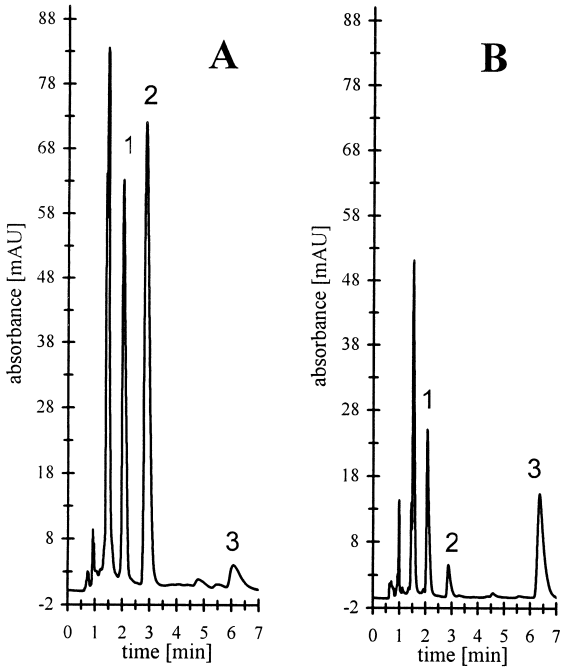

Fig. 7. Chromatograms of a representative urine sample derivatized with CMQT: (A), after treatment with TNBP; (B), without treatment with TNBP. Peaks: X, unknown; 1, internal standard; 2, cysteine (total, 141.0; free 9.6 nmol/ml urine); 3, excess of CMQT. Analytical procedure as described in Sections 2.3 and 2.4.

3.4. Validation

3.4.1. Linearity

The linearity between the cystine concentration and the measured peak area or peak height of CSH–CMQT derivative was determined by analyzing normal urine spiked with cystine, at eight different concentrations within the range from 20 to 400 nmol/ml, and internal standard. At each concentration five replicates were assayed as described in experimental Section 2.3. The analysis was repeated on a third day. The regression equations for the concentration of cystine standard solution versus the peak area and peak height are $y = 0.0052x + 1.4807$ ($r^2 = 0.9987$) and $y = 0.0050x + 1.4715$ ($r^2 = 0.9990$), respectively, for day 1. These equations for day 3 are $y = 0.0033x + 0.9922$ ($r^2 = 0.9990$), and $y = 0.0035x + 0.9991$ ($r^2 = 0.9987$), respectively.

3.4.2. Dilution parallelism

Two urine samples were diluted with water from 0 to 128 fold and so obtained aliquots were assayed as is described in procedure 1, experimental Section 2.3. As can be seen from Table 1, where the results are summarized, good linearity was obtained with both urine samples.

3.4.3. Recovery and imprecision

The recoveries were measured by the addition of the cystine standards to water and urine samples of known concentration of endogenous total cysteine from an equivalent urine matrix. After addition of internal standard the samples were then processed through whole procedure 1, described in Section 2.3. Recovery and imprecision, expressed as relative standard deviation values, were calculated and are displayed in Table 2. We have chosen cystine as a primary calibrator. Cystine can be commercially obtained with a high nominal purity and does not decompose on storage.

3.4.4. Detection and quantitation limits

The lower limit of detection and quantitation of cysteine were estimated by analysis of standard solutions of decreasing concentrations and normal urine. They were established to be 0.09 nmol/ml (1.8 pmol on column) and 0.28 nmol/ml (5.7 pmol on column), and 0.23 nmol/ml (4.6 pmol on column) and 0.77 nmol/ml (15.4 pmol on column), respectively. At these concentrations the signal-to-noise ratio was three and nine, respectively.

3.4.5. Stability of the 2-S-quinolinium derivatives

Derivatives of cysteine and internal standard were kept in a urine sample, acidified after derivatization with 3 *M* PCA to pH 1.5 (indicator paper), at room temperature. No significant change in peak heights was noted after 24 h. Results of analysis are shown at Fig. 8.

3.4.6. Internal standard

In order to minimize the contributions of sample preparation, injection variations and column deterioration to the final results, the internal standard approach was used. 2-Mercaptopropionic acid, 3-mercaptopropionic acid, thiomalic acid and thioglycolic acid were tested as candidate internal standards. All these compounds, with the exception of 3-mercaptopropionic acid, eluted too early. 3-

Table 1
Dilution parallelism

Urine: water ratio (v/v)	Total cysteine (nmol/ml)			$\frac{\text{Observed}}{\text{Calculated}} \cdot 100\%$
	Observed, $n=4$		Calculated	
	Mean (SD)	RSD (%)		
Urine 1				
1: 0	191.47 (3.34)	1.7	191.5	100.0
1: 1	97.94 (0.59)	0.6	95.7	102.3
1: 4	45.29 (2.54)	5.6	47.9	94.6
1: 8	23.32 (0.65)	2.8	23.9	97.4
1: 16	11.59 (0.27)	2.3	11.9	96.8
1: 32	6.04 (0.13)	2.1	5.9	101.1
1: 64	2.88 (0.15)	5.3	3.0	96.3
1: 128	1.43 (0.05)	3.2	1.5	96.1
Mean (SD)	97.8 (2.7)			
Urine 2				
1: 0	141.01 (3.37)	2.4	141.0	100.0
1: 1	68.49 (0.63)	0.9	70.5	97.1
1: 4	35.01 (0.63)	1.8	35.2	99.3
1: 8	16.81 (0.39)	2.3	17.6	95.4
1: 16	9.06 (0.38)	4.2	8.8	102.8
1: 32	4.59 (0.15)	3.3	4.4	104.2
1: 64	2.18 (0.15)	6.8	2.2	98.9
1: 128	1.13 (0.05)	4.5	1.1	102.5
Mean (SD)	100.1 (3.0)			

Mercaptopropionic acid, appearing as a symmetric peak, eluted near cysteine, in a position free of other peaks. Therefore, the disulphide form of this compound was adopted in subsequent investigations. The internal standard was carried out through all steps of the assay procedure.

3.4.7. Application to authentic urine samples

The optimized CMQT–HPLC procedure was applied to the analysis of urine samples of an apparently healthy volunteers, 24–30 years old (men and women). Urine samples were analyzed without delay just after receiving. Total urinary cysteine was determined in 15 samples; for five of them, free cysteine was also assayed (Table 3). In general total cysteine was higher in men than in women, with one exception, however, which was a woman (subject 3) showing a very high level of total cysteine and a very low, free cysteine fraction.

Table 2
Recoveries and within-analysis imprecision of cysteine added to urine and water as cystine, $n=5$

Cystine added (nmol/ml)	Recovery (%)		RSD (%)	
	Water matrix	Urine matrix	Water matrix	Urine matrix
20	99.1	98.2	1.4	2.0
40	99.9	104.0	1.3	1.5
80	101.1	98.6	1.9	0.7
400	101.6	103.1	0.8	1.1
800	100.9	99.0	0.5	1.2

Table 3
Total and free cysteine in authentic urine samples

Subject	Sex (F, M)	Age (years)	Total cysteine		Free cysteine		
			Found (nmol/ml)	RSD (%)	Found (nmol/ml)	RSD (%)	Of total (%)
1	M	29	141.0	2.0	9.6	2.7	6.8
2	M	28	181.9	2.1	23.7	2.9	13.0
3	F	24	190.3	1.2	5.5	4.3	2.9
4	F	28	72.9	1.4	5.6	4.1	7.7
5	F	30	65.8	1.4	4.4	3.9	7.6

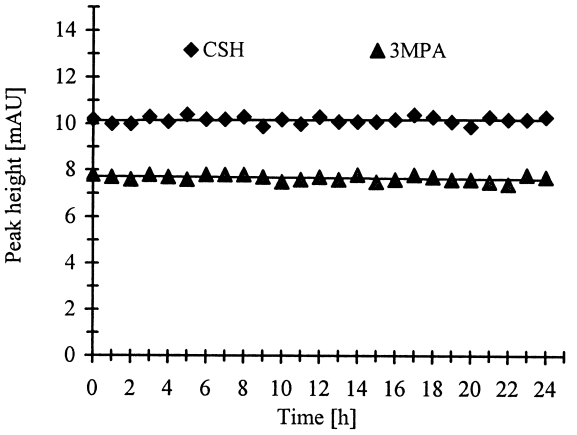

Fig. 8. Stability of the *S*-quinolinium derivatives in standard urine solution at room temperature. Experimental conditions as described in Sections 2.3 and 2.4.

4. Conclusion

We have developed a CMQT–HPLC technique which make it possible to detect and quantitate total and free cysteine in urine in humans. Reduction with a suitable reagent such as tri-*n*-butylphosphine is essential for measuring total cysteine because it readily forms disulphide bonds with itself, homocysteine and other endogenous and exogenous thiols present in urine. The protocol described is a robust, user friendly, rapid assay, suitable for clinical and pediatric settings. The use of CMQT as a derivatization reagent, carefully optimized HPLC separation conditions and an appropriate internal standard, 3-mercaptopropionic acid, has led to substantial improvements over our previously reported [18] method. The improvements encompass much better resolution, higher sensitivity, better accuracy (99.1–101.6 versus 91–114%), lower imprecision (0.7–2.0 versus 1.1–5.2%) and shorter separation time (6 versus 30 min). Our present method gives chromatograms showing desired peaks (analyte, internal standard and reagent excess) and only one unwanted peak eluting close to solvent front. The method can compete with widely recognised methods such as mBrB–HPLC [9,10] or DBDF (SBDF)–HPLC [11,12] known for their inherent sensitivity as well as for some drawbacks associated with their application to analysis of biological thiols [11,19–21].

Our method should find application in routine analysis of cysteine in urine in experimental and clinical laboratories.

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