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CYSTEINYLGLYCINE IN URINE DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Using N-(1-pyrene)maleimide as a reagent for thiol compounds and high-performance liquid chromatography with fluorometric detection, we have identified cysteinylglycine as an endogenous compound in dithiothreitol-reduced urine. In a quantitative method developed for cysteinylglycine, reduction of urinary disulphides was effected by dithiothreitol at pH 6. The pH was then brought to 1.5 and excess dithiothreitol together with acid thiols was extracted with water-saturated ethyl acetate. After derivatization the concentration was determined by reversed-phase liquid chromatography. Precision of the method (C.V. = 6.5%) and analytical recovery (86 ± 6.4%) were satisfactory. The urinary excretion of cysteinylglycine was 7.4 ± 2.3 μmol/l (mean ± S.D.) in eight healthy subjects.

INTRODUCTION

A number of thiol compounds are present in animal cells and take an active part in metabolism. One of the best known of these thiols is glutathione which is present in high amounts in, for example, liver cells and erythrocytes, but is present in much lower concentrations extracellularly [1]. According to the γ-glutamyl cycle the first step in glutathione breakdown is catalysed by γ-glutamyltranspeptidase, which removes the γ-glutamyl moiety of glutathione and transfers it to an acceptor amino acid [2]. The remaining cysteinylglycine is then split to cysteine and glycine in a reaction which may be very rapid. Thus, after injection of radioactively labelled glutathione to rats [3], the radioactivity within the tissue was essentially recovered as labelled glycine, and no radioactive cysteinylglycine was observed in the blood or tissues examined. However, when isolated renal tubules were incubated with radioactive glutathione disulphide, a third radioactive compound in addition to glutathione disulphide and glycine appeared, which was identified as cystinyldiglycine, the symmetrical disulphide of cysteinylglycine (Fig. 1).

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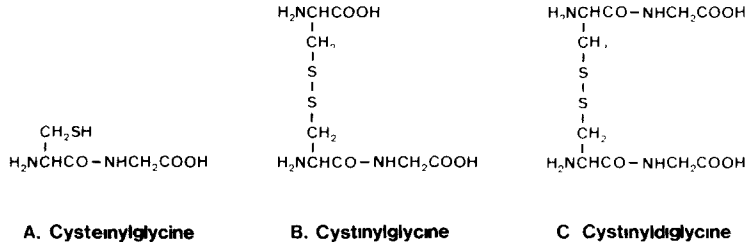

Fig. 1. Structure of (A) cysteinylglycine, (B) cystinylglycine (mixed disulphide of cysteine and cysteinylglycine), and (C) cystinyldiglycine (symmetric disulphide of cysteinylglycine).

The compound cystinylglycine (Fig. 1), which probably also is a metabolite of glutathione [4], occurs regularly in blood plasma, but has not been observed in urine [5]. Both cystinyldiglycine and cystinylglycine give the thiol cysteinylglycine after reduction of their disulphide bridges.

We have previously developed a highly sensitive method for the determination of the acid thiols mercaptoacetate and N-acetylcysteine in urine [6]. After reduction of disulphide bridges and clean-up of the liberated acid thiols from the urine, the thiols were reacted with a non-fluorescent maleimide compound [7] thereby forming fluorescent derivatives which could be separated by high-performance liquid chromatography (HPLC) and quantified by fluorimetry. Using similar techniques we have now identified the compound cysteinylglycine in reduced urine and developed an HPLC method for its determination.

EXPERIMENTAL

Materials

3-Mercaptopropionic acid was obtained from E. Merck (Darmstadt, F.R.G.) and cystinyldiglycine $(Cys\text{-}Gly)_2$ was from Serva (Heidelberg, F.R.G.). Dithiothreitol was obtained from Sigma (St. Louis, MO, U.S.A.) and the anion exchanger AG1-X4 (100—200 mesh, Cl^-) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). The organomercurial adsorbent *p*-acetoxymercurianiline—Sepharose 4B (PAMAS) was prepared according to the procedure of Sluyterman and Wijdenes [8]. N-(1-Pyrene)maleimide was a product of Fluka (Buchs, Switzerland). We purified the substance by chromatography on a Lobar LiChroprep Si-60 column, size B (Merck). For this procedure we used an Eldex E-120-S-2 (Eldex Labs., Menlo Park, CA, U.S.A.) pump and a mobile phase of toluene—acetone (9:1, v/v). After equilibration of the column, 5 mg of N-(1-pyrene)maleimide, dissolved in 3 ml of the mobile phase were transferred to the column and eluted at a flow-rate of 1.0 ml/min. Detection was by ultraviolet (UV) absorption at 339 nm (Spectromonitor III, LDC, Riviera Beach, FL, U.S.A.). Fractions (5 ml) were collected and the relevant fractions evaporated; the N-(1-pyrene)maleimide was dissolved in ethanol—acetone (1:1, v/v) and its concentration determined from its UV absorption at 339 nm. A stock solution, 1.0 mmol/l, was prepared in ethanol—acetone (1:1, v/v) and stored in the refrigerator.

Apparatus

We used a Constametric III pump from LDC, a Rheodyne (Cotati, CA,

U.S.A.) Model 7125 sample injector with a 100-μl sample loop, and a Perkin-Elmer (Beaconsfield, U.K.) Model 3000 spectrofluorometer with an LC-cell accessory. Excitation wavelength was 342 nm (band width 15 nm) and emission wavelength was 396 nm (band width 20 nm).

The analytical column was a Supelcosil LC-8 column (250 × 4.6 mm), particle size 5 μm, from Supelco (Bellefonte, PA, U.S.A.). In order to protect the analytical column, a precolumn packed with Solvecon Silica, particle size 37—53 μm (Pierce, Rockford, IL, U.S.A.), was put in line between the pump and the injector. The columns were jacketed and held at constant temperature (25 or 35°C) by connection to a circulating water-bath in order to obtain reproducible retention times. The mobile phase was prepared from phosphoric acid (85%, Merck) diluted with distilled water to a concentration of 50 mmol/l and mixed with methanol in the proportions 11:9 (v/v).

Urine collection

Urine was collected for 24 h in plastic bottles containing 5 ml of thymol, 0.7 mol/l, in isopropanol as a preservative [9].

Reduction of urinary disulphides with thiopropyl-Sepharose 6B

We followed the procedure described by Hannestad and Sörbo [10] for reduction of urinary disulphides. Thus we added 0.2 ml of Na_2EDTA, 0.13 mol/l, to a 5-ml aliquot of urine, adjusted the pH to 9.8—10.0 with ammonia, 5 mol/l, and made up the volume to 6 ml with water. Then 1 ml of thiopropyl-Sepharose 6B suspension containing 20 μmol of SH-groups per ml gel was added and reduction was effected during 30 min mixing. Acidification was then done to pH 3.5—4.0 by the addition of 1 ml of acetic acid, 4 mol/l, and the sample was centrifuged. From this solution samples were taken either for derivatization or further clean-up. For derivatization 500 μl were transferred to new tubes; 5 ml of carbonate buffer, 50 mmol/l, pH 9.0, containing Na_2EDTA, 10 mmol/l, were added followed by 250 μl of sodium hydroxide, 1 mol/l, and 500 μl of N-(1-pyrene)maleimide, 1 mmol/l.

Clean-up of reduced urine with PAMAS and anion exchanger (AG1-X4)

From the urine reduced by thiopropyl-Sepharose, an amount (usually 5 ml) containing less than 2 μmol of thiols as measured by the method of Rootwelt [11] was adsorbed on a PAMAS column (bed dimension 1.3 × 0.7 cm) and after washing with 2 ml of water the adsorbed thiol compounds were eluted with 3 ml of 3-mercaptopropionic acid, 10 mmol/l, into tubes containing 0.2 ml of Na_2EDTA, 0.18 mol/l. We then adjusted the pH to about 7, and applied the sample to a 2.6 × 0.5 cm AG1-X4 (Cl^-) column (equilibrated with imidazole solution, 50 mmol/l, adjusted to pH 7.0 with hydrochloric acid), which retains the 3-mercaptopropionic acid. The effluent was collected in a tube containing 0.2 ml of Na_2EDTA, 0.18 mol/l, together with a further 1 ml of imidazole buffer applied to the column. A 500-μl aliquot of the combined effluents was mixed with 5 ml of carbonate buffer, 50 mmol/l, pH 9.0, containing Na_2EDTA, 10 mmol/l, followed by 500 μl of N-(1-pyrene)-maleimide, 1 mmol/l.

Quantitative analysis of cysteinylglycine

To a 5-ml aliquot of urine were added 200 μl of Na_2EDTA, 0.13 mol/l, and 500 μl of dithiothreitol, 1 mol/l. Standard was prepared daily as an aqueous solution of cystinyldiglycine, 10 μmol/l, corresponding to a cysteinylglycine concentration of 20 μmol/l after reduction. A 5-ml volume of this solution was mixed with Na_2EDTA and dithiothreitol in the same way as urine. We then adjusted the pH of the sample to 6.0 with sodium hydroxide, 1 mol/l, or hydrochloric acid, 1 mol/l, and made up to a final volume of 6.0 ml with water. After mixing, the tubes were left at room temperature for 1.5 h. With hydrochloric acid, 6 mol/l, the pH was then adjusted to 1.5 ± 0.1 and water was added to a final volume of 6.2 ml. From this solution 1 ml was shaken horizontally for 5 min with 5 ml of ethyl acetate saturated with water. The phases were separated by centrifugation and the organic phase was discarded. After four-fold extraction the water phase was evaporated at 40° C in a Vortex-Evaporator (Buchler, Fort Lee, NJ, U.S.A.). Then 10 ml of carbonate buffer, 50 mmol/l, pH 9.0, containing Na_2EDTA, 10 mmol/l, were added, the pH was adjusted to 9.0 ± 0.1 with sodium hydroxide, 1 mol/l, and the volume made up to 10.2 ml with water. From this solution 5 ml were derivatized with 500 μl of N-(1-pyrene)maleimide, 1 mmol/l, and HPLC was performed as described below.

Derivatization and high-performance liquid chromatography

After mixing of sample and standard solutions with derivatization reagent at pH 9.0 in tubes with PTFE-lined screw-caps, the tubes were placed in a water-bath at 37° C for approximately 20 h before five-fold dilution with the mobile phase and loop injection on the HPLC column. In all experiments we used a flow-rate of 1 ml/min. In initial studies we used a column temperature of 35° C, which gave satisfactory separation. However, at this temperature a peak from remaining dithiothreitol, used in the final method, interfered with the cysteinylglycine peak. Separation of these peaks was accomplished by lowering the temperature to 25° C.

RESULTS

Chromatography of reduced urine

Fig. 2 shows a chromatogram of thiopropyl-Sepharose 6B-reduced urine. From earlier work we knew the identification of 3-mercaptolactate, N-acetylcysteine, mercaptoacetate and cysteine. Among the unknown peaks there was one peak eluting between N-acetylcysteine and mercaptoacetate with the same retention time as cysteinylglycine. Additional peaks were seen in the void volume and with retention times around 20 min.

Anion-exchange experiments

Cysteinylglycine is a dipeptide with amphoteric character, and thus its properties differ markedly from the properties of acid thiols. In contrast to these it is retained by a cation exchanger, and we found that it disappeared completely when reduced urine was purified for determination of acid thiols [6]. To purify cysteinylglycine in urine from acid thiols, thiopropyl-Sepharose-

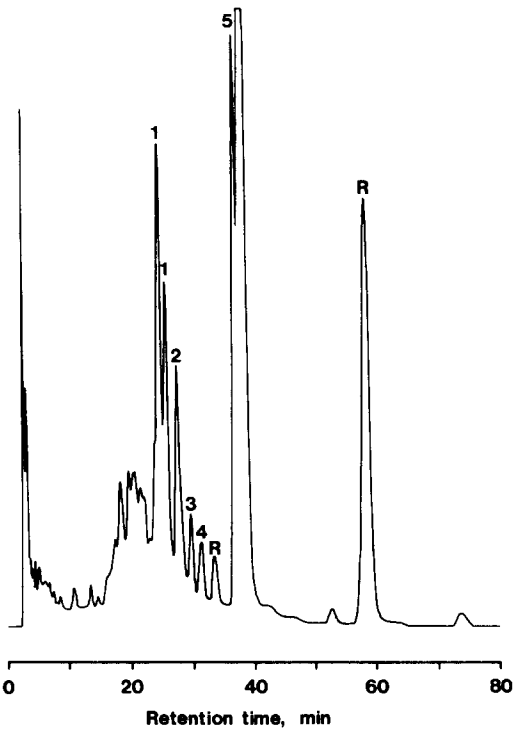

Fig. 2. Chromatogram of a urine sample after reduction with thiopropyl-Sepharose 6B. No prepurification was done before derivatization. Peak identification: 1 = 3-mercaptolactate as a double peak, 2 = N-acetylcysteine, 3 = cysteinylglycine, 4 = mercaptoacetate, 5 = cysteine as a double peak, and R = reagent peaks. Column temperature 35°C.

reduced urine was transferred to a PAMAS column, the column was washed with water, and elution was then effected by 3-mercaptopropionic acid. The sample adjusted to pH 7.0 was then transferred to an AG1-X4 column equilibrated with an imidazole buffer, 50 mmol/l, pH 7.0, a pH at which acid thiols like 3-mercaptopropionate should be retained. The first effluent together with 1 ml of washing with imidazole buffer was derivatized. On HPLC we found a chromatogram (Fig. 3) with only a few peaks, among them cysteinylglycine and cysteine. However, although cysteinylglycine was highly purified from acid thiols by this procedure the recoveries of cysteinylglycine were not reproducible. This may be due to incomplete recovery from the ion exchanger or to instability of cysteinylglycine at the high pH used in the reduction step. From other studies it has been claimed [12] that cysteinylglycine is subject to non-enzymatic oxidation at pH values above 7, and we therefore abandoned this procedure as a quantitative method for cysteinylglycine in urine.

Method for determination of cysteinylglycine in urine

Since cysteinylglycine is unstable at alkaline pH we finally chose to reduce urine with dithiothreitol at pH 6.0. Before the ensuing extraction of dithiothreitol with ethyl actate the pH was adjusted to 1.5, a pH at which cysteinylglycine remains in the water phase in contrast to weakly acid thiols like 3-mercaptolactate. In spite of four extractions, traces of dithiothreitol

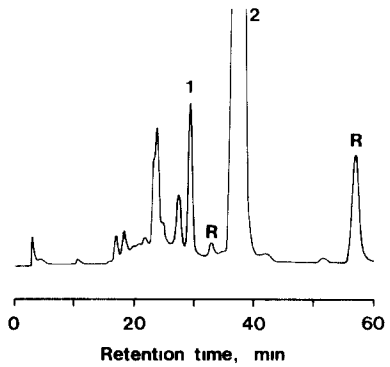

Fig. 3. Chromatogram of thiopropyl-Sepharose 6B-reduced urine purified on PAMAS and AG1-X4 columns. Peak identification: 1 = cysteinylglycine, 2 = cysteine, and R = reagent peaks. Column temperature 35° C

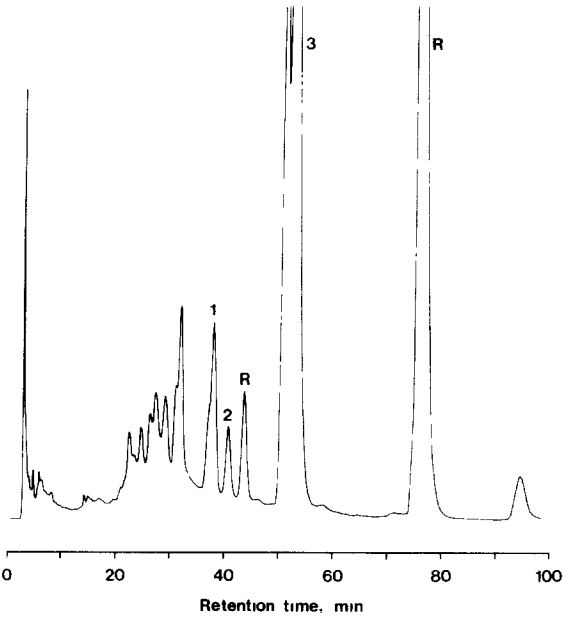

Fig. 4 Chromatogram of dithiothreitol-reduced urine. Peak identification· 1 = dithiothreitol, 2 = cysteinylglycine, 3 = cysteine, and R = reagent peaks. Column temperature 25° C

appeared in the chromatogram close to cysteinylglycine by the standard HPLC procedure. This peak, however, was well separated from the cysteinylglycine peak when the chromatography was performed at 25° C instead of 35° C. The resulting chromatogram is shown in Fig. 4. Well resolved peaks for cysteinylglycine and cysteine were seen. This procedure was adopted for quantitative determination of cysteinylglycine in urine.

Precision and recovery of the quantitative method

Duplicate determinations were performed on seven urine samples. They had a mean cysteinylglycine concentration of 6.3 μmol/l. From the duplicate determinations the S.D. was calculated as 0.41 μmol/l, which gave a C.V. of

6.5%. Recovery was determined from urinary samples to which cystinyldiglycine was added to increase the cysteinylglycine concentration by 20 μmol/l. The recovery was determined as 86 ± 6.4%.

Normal values

Urine samples were collected from eight healthy members of staff. They had a mean urinary excretion of 7.4 ± 2.3 μmol per 24 h. The range was 5.0 to 10.8 μmol per 24 h.

DISCUSSION

By the experiments presented here we have identified cysteinylglycine as an endogenous compound in reduced urine. It may be of interest to summarize the criteria we have used to make this statement. Thus, with the HPLC we found that the retention time of authentic cysteinylglycine corresponded perfectly with the peak in question from urine. This was also confirmed as an increase of the peak when cysteinylglycine was added to the urine. The retention time also changed in a similar way for standard and the urinary compound when the temperature of the chromatography procedure was changed. Further, the compound was adsorbed by the organomercurial adsorbent used in the clean-up of other thiol compounds [6, 10] from urine, and was competitively eluted by 3-mercaptopropionate. Its reaction with N-(1-pyrene)maleimide is a similar sign of thiol property. The behaviour of the endogenous compound in cation- and anion-exchange experiments was as expected and similar to that of standard solution. Also pH-stability experiments performed (data not shown) showed identical properties for the endogenous compound and the standard.

With the present method the amount of cysteinylglycine is quantified as free thiol after reduction of disulphides in urine. The method thus determines the sum of free cysteinylglycine, cystinyldiglycine $(Cys\text{-}Gly)_2$ and cystinylglycine (mixed disulphide of cysteine and cysteinylglycine). Also other mixed disulphides of cysteinylglycine may be found in urine, and still other non-disulphide compounds which can be reduced to cysteinylglycine may exist in human urine.

Recently a scheme for the renal metabolism of glutathione was suggested by Jones et al. [4]. Glutathione is first oxidized to glutathione disulphide and thereafter a first γ-glutamyl residue is removed. This is effected by the enzyme γ-glutamyltransferase. Then either the remaining γ-glutamyl residue is removed, which gives cystinyldiglycine, or a glycine moiety is removed, leaving the disulphide of cysteine and glutathione (preferred pathway). Subsequent removal of a γ-glutamyl residue from the latter disulphide or removal of glycine from cystinyldiglycine gives cystinylglycine. Cleavage of the disulphide bridge of the latter compound or of cystinyldiglycine gives cysteinylglycine.

Hahn et al. [3] incubated radioactively labelled glutathione disulphide with renal tubules from rats. They clearly detected cystinyldiglycine, but they could not show the presence of cysteinylglycine in renal tissue after injection of glutathione. These findings are at variance with the findings of a cysteinylglycine dipeptidase [13], which may degrade cyst(e)inylglycine and cystinyldiglycine rapidly.

It has been shown that cystinylglycine is present in human serum at a concentration of about 10 μmol/l [5], but the compound was not demonstrated in urine. The findings in plasma were confirmed by Perry and Hansen [14], and they further showed that it was absent in plasma of a patient with pyroglutamic acidaemia, and its concentration was greatly reduced in homocystinuria. Small amounts (0.23 μmol/l) of cysteinylglycine have been reported to occur in human serum [15]. The present work shows the presence of cysteinylglycine or compounds that can be reduced to this compound in urine. From the foregoing discussion it is probable that it comes from degradation products of glutathione. Further studies will show the exact nature of the compound in urine.

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