High-performance Liquid Chromatographic Analysis of Urinary Hydroxylysyl Glycosides as Indicators of Collagen Turnover

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Hydroxyproline is the urinary metabolite commonly measured to evaluate collagen turnover, but the assay for the component does not provide quantitative information on collagen breakdown. In fact, only 10–25% of collagen hydroxyproline is excreted in the urine[1] and further urinary hydroxyproline may derive from collagen of different tissues such as bone or soft tissues.

The glycosides of hydroxylysine (HLG), α-1,2-glucosylgalactosyl-*O*-hydroxylysine (GGH) and β-1-galactosyl-*O*-hydroxylysine (GH) appear to be better indicators of total collagen turnover than hydroxyproline.[2] Moreover, quantitative evaluation of urinary HLG indicates that collagen turnover is in fact 2–4-fold higher than that calculated from hydroxyproline measurements and accounts for 50–100% of degraded collagen.[3] HLG excretion is not significantly affected by diet[2] and by measuring GH and GGH in urine it is also possible to evaluate the metabolic turnover of different collagen, *e.g.*, from bone and skin. In fact, the ratio of the amounts of the two urinary metabolites is influenced by the type of collagen metabolised and by the age of the subject.[3]

Numerous methods for determining urinary HLG have been reported.[2] The procedure generally consists in HLG separation by ion-exchange chromatography, followed by their quantitation using the ninhydrin reaction or the orcinol - sulphuric acid assay for hexoses.[4] The amino acid analyser is so far the instrument of choice for separating and determining these metabolites in urine. In this paper, we describe a rapid and more sensitive method for measuring HLG in urine by converting the amino groups of hydroxylysine into fluorescent derivatives with dansyl chloride prior to their separation by reversed-phase high-performance liquid chromatography (HPLC).

Experimental

Chemicals

Reagents obtained from commercial suppliers were of analytical-reagent grade. Acetonitrile was purchased from BDH Chemicals (Poole, Dorset, UK) and 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride; Dns-Cl) from Sigma (St. Louis, MO, USA). Standards of GH and GGH were a generous gift from the Institute of Biological Chemistry of the University of Pavia (Italy), where they were prepared from sponges.[5]

Reagents

α-1,2-*Glucosylgalactosyl-O-hydroxylysine solution*, 116 nmol ml^{-1}.

β-1-*Galactosyl-O-hydroxylysine solution*, 74 nmol ml^{-1}.

Sodium carbonate solution, 0.3 M.

Dansyl chloride solution in dimethyl ketone, 10 mg ml^{-1}.

Buffer A. Sodium acetate (0.05 M, pH 6.3) + acetonitrile (12.5%) + propan-2-ol (5%).

Buffer B. Sodium acetate (0.05 M, pH 6.5) + acetonitrile (50%) + propan-2-ol (1%).

Procedure

Urine collection

The urine specimens (24 h) contained 0.1% of boric acid to prevent growth of bacteria.

Sample preparation

HLG standards and aliquots of urine were derivatised according to the method of Gray.[6] Briefly, 50 μl of an aqueous solution of HLG (5.8 nmol of GGH or 3.7 nmol of GH) or 50 μl of urine were added to a mixture of 50 μl of 0.3 M Na_2CO_3 solution and 100 μl of Dns-Cl in dimethyl ketone (10 mg ml^{-1}). The mixture was incubated at 60 °C for 30 min and 20 μl of the mixture were then injected into the column. The urine specimen did not require any hydrolytic pre-treatment, as already reported by Askenasi.[4]

HPLC procedure

HPLC separation was carried out with a Beckman Model 344 instrument, connected with a fluorimeter, using an excitation wavelength of 366 nm and an emission wavelength of 490 nm. The area of the peaks was calculated by an HP 3390 automatic integrator. Reversed-phase HPLC was performed using a 250 × 4.6 mm i.d. column of Ultrasphere-ODS (C_{18}) (5 μm); the two solvent systems used for the stepwise gradient were buffer A and buffer B. The flow-rate was maintained at 1 ml min^{-1}.

Results

In a series of preliminary experiments, the optimum stepwise gradient necessary for the full resolution of HLG was determined.

The elution patterns of the GGH and GH standards are shown in Fig. 1(*a*). The former standard (1.07 nmol injected) elutes as a double peak at 43.88 ± 0.37 and 44.19 ± 0.37 min [mean of ten experiments ± standard deviation (S.D.)]. The latter standard, GH (0.67 nmol injected), also elutes as a double peak at 49.04 ± 0.54 and 50.51 ± 0.33 (mean of ten experiments ± S.D.). Fig. 1(*b*) shows the elution profile of a 24-h urine sample from a boy aged 14. Many peaks can be seen, but under the experimental conditions reported, four peaks elute with the same profiles as the retention times of GGH and GH doublets. Addition of HLG standards (1.07 nmol of GGH and 0.67 nmol of GH) to the urine sample causes an increase of the profile peaks, as shown in Fig. 1(*c*).

The retention time and peak areas are reproducible for the standards, whereas the other chromatographic peaks are independent from those of the major derivatives. On the basis of the fluorescence intensity, there was 100% recovery of the injected standards.

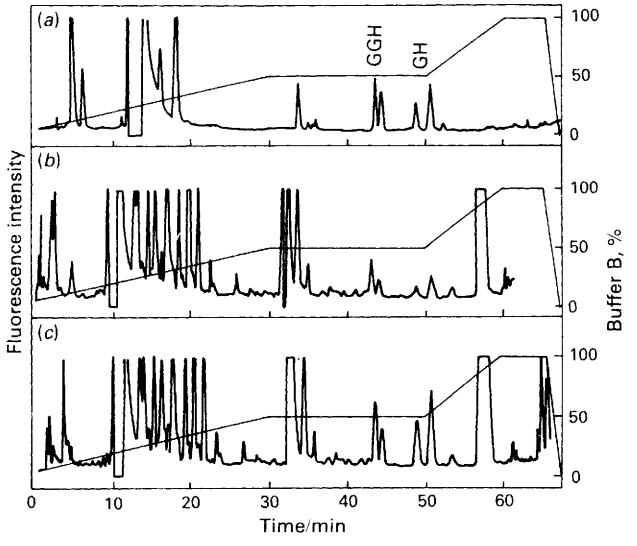

Fig. 1. HPLC elution profile of glycosides of hydroxylsine. (*a*) A mixture of 1.07 nmol of GGH and 0.7 nmol of GH was injected; (*b*) sample of 24-h urine; and (*c*) 1.07 nmol of GGH and 0.7 nmol of GH were added to the sample of urine before conversion into dansyl derivatives

By statistically evaluating the imprecision of the peak-area measurements and the batch imprecision of urine analysis, a coefficient of variation of 5% was obtained. Analysis of the correlation between peak areas and amounts of GGH and GH in the range 0–2 nmol provides the equations $y = 0.40x + 0.05$ and $y = 0.59x + 0.05$, respectively, with $r = 0.99$ in both instances.

Discussion

Under the experimental conditions reported, HLG standards are well separated. With a sample of urine, four peaks appear that are identical with the standards with respect to both retention time and peak shape and are identified as GGH and GH.

The fact that each collagen metabolite is marked by a double peak suggests the formation of mono- and di-dansyl derivatives of the compound. Based on the lipophilic properties of the two derivatives, it is suggested that the first peak of each pair is the mono-derivative and the second the di-derivative. As shown in the elution profile, the integrated area of the first peak of GGH is higher than that of the second, whereas the opposite applies to the GH peaks. This means that with the GGH doublet less of the molecule is di-dansylated in comparison with the GH doublet. This is not surprising if one considers that the glucidic moiety of GGH is larger than that of GH, leading to steric hindrance. Hence two different reaction rates for the formation of the dansyl derivatives could occur, which would explain the different slopes of the calibration graphs. The slope of the GH calibration graph is steeper than that of GGH, as there is more fluorescence per mole in the former than in the latter.

The sensitivity of the method is of the order of picomoles, as at 0.5 nmol of HLG the integrated peak areas are still high.

The determination of GGH and GH in urine is more rapid than with other procedures, as the sample preparation step[2] does not appear to be essential. Comparing the data obtained by other workers using conventional chromatographic techniques, the values we have obtained are of the same order of magnitude as those reported by Askenasi,[7] Segrest and Cunningham,[3] Kakimoto and Akazawa[8] and Cetta *et al.*[9]

Finally, HPLC equipment is much less expensive than an amino acid analyser and also offers a wider range of analytical applications to potential users.

In conclusion, the method described here appears to be reliable, simple and rapid and can be applied routinely to monitor the turnover rate of collagen in clinical conditions where this protein is involved.

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