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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PREPARATION OF GALACTOSYL-HYDROXYLYSINE, A SPECIFIC BONE COLLAGEN MARKER

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## SUMMARY

Galactosyl-hydroxylysine, a specific bone collagen marker, has been prepared directly from human urine samples by high-performance liquid chromatography (HPLC) on a preparative column. The compound is the didansyl derivative, as proved by HPLC and mass spectrometry under fast atom bombardment conditions. Since this compound is not commercially available, the procedure reported appears to be the simplest way to prepare it, which is necessary to measure the urinary excretion of this collagen metabolite by HPLC.

## INTRODUCTION

The monoglycoside of hydroxylysine, galactosyl-hydroxylysine (GH), is one of the most suitable diagnostic markers in post-menopausal osteoporosis [1], a widely diffused disease characterized by loss of bone mass accompanied by bone fractures. The predictive value of GH determination in such a disease, in

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terms of sensitivity and specificity, is practically the same as that obtained by measuring bone mineral density using quantitative computed tomography (QCT) [1].

GH is almost completely excreted in urine. Its rate of excretion is not affected by diet [2] and it is inversely correlated with bone mineral density [3]. The method proposed for GH measurements in urine is based on the high-performance liquid chromatographic (HPLC) separation of the dansyl derivative. For such an approach, a specific standard is required to identify the GH peak [4]. Since GH is not commercially available, the following alternatives can be used for its preparation: (a) alkaline hydrolysis of a number of collagens or basement membrane preparations [2]; (b) organic synthesis of the compound [5].

Both procedures are time-consuming and provide a limited amount of GH in the racemic form [2,5], which leads to a double peak in the elution profile on reversed-phase HPLC [4]. A quick procedure to prepare a standard of GH is thus a necessary requirement for a wider adoption of the GH test in bone diseases.

This paper describes a method to prepare a standard of GH directly from urine of human subjects, by using a preparative column with the HPLC instrument. Satisfactory amounts of the compound were obtained, and not in the racemic form. The standard is didansylated and can be immediately used or lyophilised and stored in deep freezer. The unambiguous identification of the compound is achieved by chromatography and by mass spectrometry (MS) in the fast atom bombardment (FAB) mode [6].

## EXPERIMENTAL

### *Chemicals*

Reagents obtained from commercial suppliers were of analytical grade. Acetonitrile and propan-2-ol were purchased from Riedel-de-Haen (Seelze, F.R.G.); 5-dimethylaminonaphthalene 1-sulphonyl chloride (dansyl chloride; Dns-Cl) and D,L-hydroxylysine were purchased from Sigma (St. Louis, MO, U.S.A.). Neither D- nor L-hydroxylysine is commercially available.

GH was a generous gift from the Institute of Biological Chemistry of the University of Pavia (Pavia, Italy), where it was prepared from sponges [7].

Sodium carbonate, sodium acetate and glycerol were purchased from Carlo Erba (Milan, Italy).

### *Reagents*

The following reagent solutions were prepared: 31.2 $\mu M$ D,L-hydroxylysine; 35 $\mu M$ $\beta$-1-galactosyl-O-hydroxylysine; 0.3 $M$ sodium carbonate; dansyl chloride solution in dimethyl ketone, 10 mg/ml; sodium acetate (0.05 $M$, pH 6.3)–

acetonitrile (12.5%)–propan-2-ol (5%) (buffer A); sodium acetate (0.05 *M*, pH 6.5)–acetonitrile (50%)–propan-2-ol (1%) (buffer B).

*Urine collection*

Samples of 24-h urine, containing 0.1% (w/v) boric acid to prevent growth of bacteria, were immediately used or kept frozen at −70 °C in liquid nitrogen.

*Sample preparation*

About 4 ml of the 24-h urine were centrifuged at 200 *g* for 10 min with an ALC centrifuge, Model 4225, in order to remove sedimentable material. The supernatant was collected and dansylated in parallel with standard GH, according to the method of Gray [8]. Briefly, 500 μl of an aqueous solution of standard GH (17.5 nmol) or 500 μl of urine were mixed with 500 μl of 0.3 *M* $Na_2CO_3$ and added to 1 ml of Dns-Cl in dimethyl ketone (10 mg/ml). The mixture was incubated at 60 °C for 30 min and then filtered through an HV 0.45-μm filter (Millipore, Nihon Millipore Kogyo K.K., Yonezawa, Japan). Aliquots of the mixture ranging from 20 to 500 μl were then injected into the column.

*HPLC separation*

HPLC separation was carried out with a Beckman Model 344 instrument, with a fluorimetric detector, using an excitation wavelength of 366 nm and an emission wavelength of 490 nm. The peak areas were calculated by an HP 3390 automatic integrator.

The analytical column was a $C_{18}$ reversed-phase analytical column (3 μm particle diameter, 7.0 cm×4.6 mm I.D., Beckman Ultrasphere ODS) connected with a 20-μl loop.

The preparative column was a $C_{18}$ reversed-phase (10 μm particle diameter, 50 cm×9.4 mm I.D., Whatman, Clifton, NJ, U.S.A.) connected with a 1000-μl loop.

TABLE I

GRADIENT ELUTION PROGRAMMES

| Column | Time (min) | Flow-rate (ml/min) | Buffer B (%) | Duration (min) |
|---|---|---|---|---|
| Analytical | 0 | 1 | 10 | — |
| | 0.1 | | 45 | 10 |
| | 18 | | 100 | 3.5 |
| | 27 | | 10 | 0.1 |
| Preparative | 0 | 3 | 20 | — |
| | 0.1 | | 100 | 80 |
| | 81 | | 20 | 0.1 |

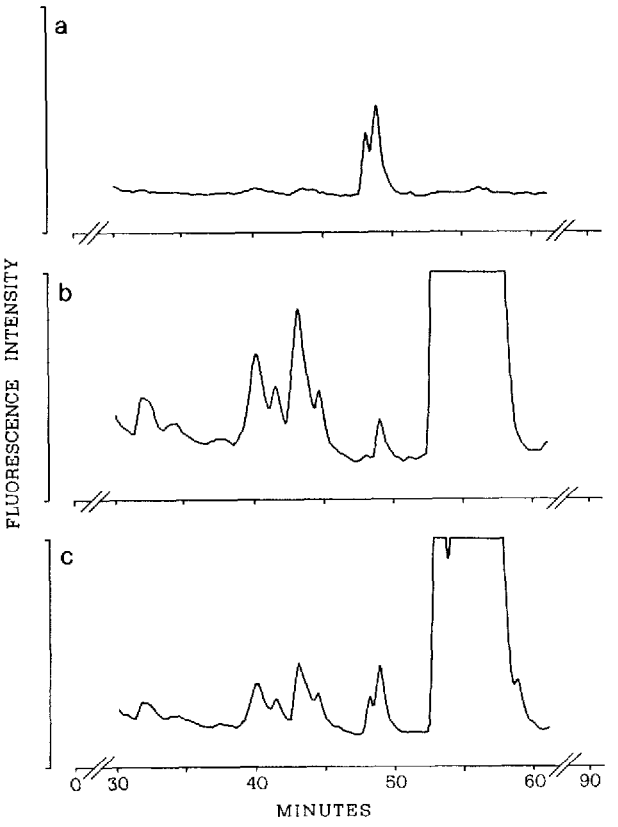


Fig. 1. HPLC elution profiles with the preparative column of (a) standard GH (0.875 nmol), (b) a 24-h urine sample and (c) standard GH (0.437 nmol) co-eluted with the urine sample.

The time programmes of the solvent gradient used with the analytical and preparative column are listed in Table I. Before use, buffers A and B were filtered through a 0.22-$\mu$m filter (GVWP 047 00 Millipore, Bedford, MA, U.S.A.).

*Mass spectrometry*

MS measurements were performed with a double-focusing, reversed-geometry VG ZAB2F instrument operating in FAB mode (8 keV Xe atoms bombarding a glycerol solution of the sample). Naturally occurring metastable transitions (daughter spectra) were obtained by magnetic field intensity/electrostatic field intensity ($B/E$) = constant linked scans [9].

## RESULTS AND DISCUSSION

On the basis of our experience in measuring urinary GH by HPLC, a stepwise gradient was set up to obtain good instrumental resolution in the region

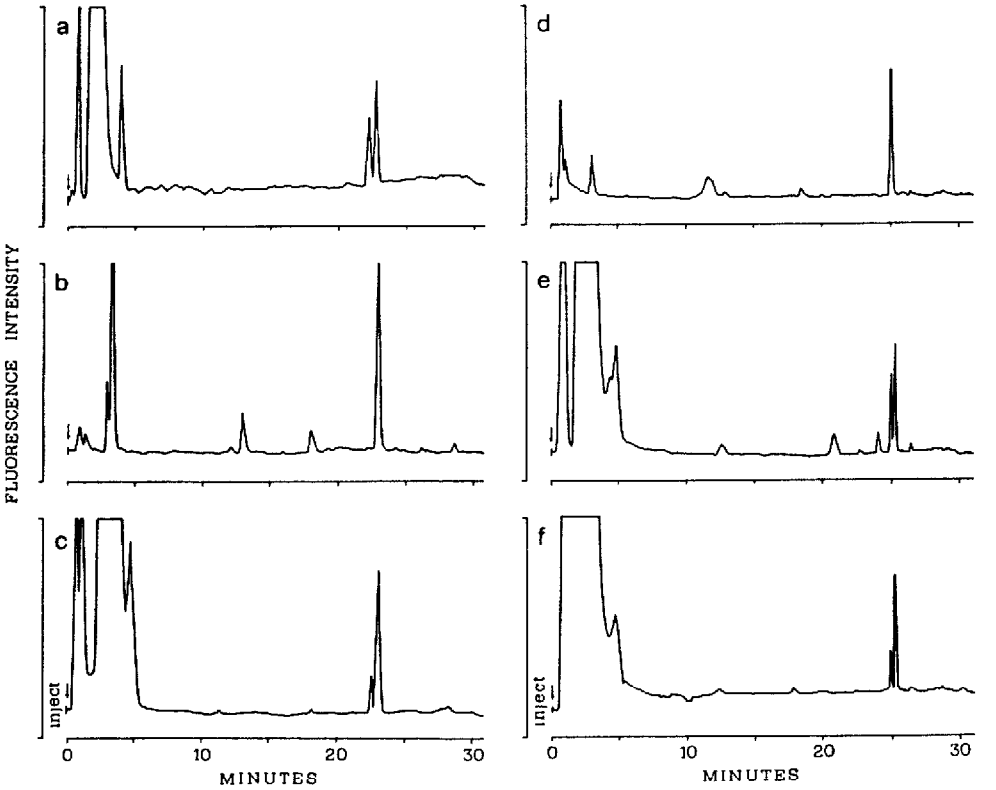


Fig. 2. HPLC elution profiles with the analytical column of (a) standard GH (0.175 nmol), (b) a fraction collected from the preparative column (see Fig. 1b), (c) standard GH co-eluted with the fraction shown in (b) (recovery = 100%), (d) the fraction shown in Fig. 1b subjected to acid hydrolysis, (e) a standard of D,L-hydroxylysine (0.156 nmol) and (f) the fraction shown in (d) co-eluted with D,L-hydroxylysine (0.078 nmol) (recovery = 90%).

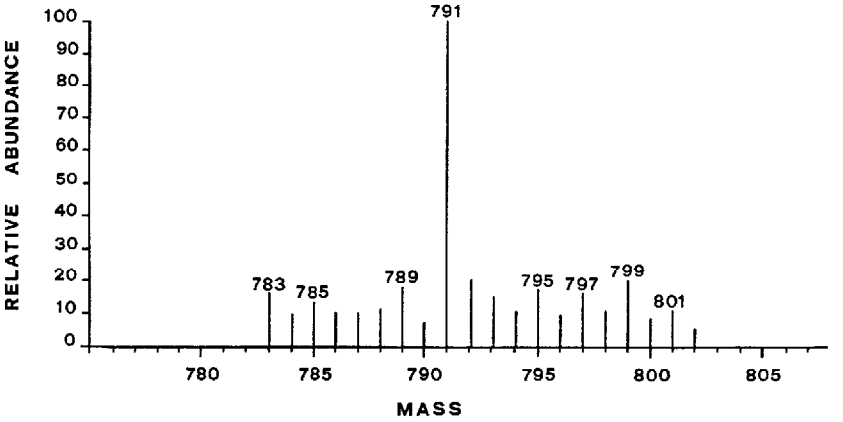


Fig. 3. Partial FAB mass spectrum of the fraction shown in Fig. 1b, showing the $(MH)^+$ species of didansyl GH at $m/z$ 791.

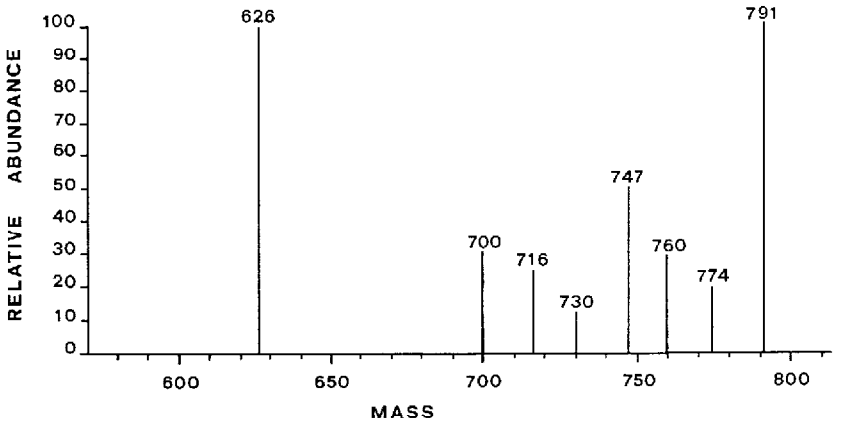


Fig. 4. $B/E$ = constant linked scan of the $(MH)^+$ ion ($m/z$ 791) of didansyl GH.

where standard GH was expected to elute. When the gradient described in Table I and the preparative column are used, standard GH appears as a doublet (Fig. 1a): the first peak is the monodansyl and the second the didansyl derivative [4].

Chromatography of a urine sample yields a peak with the same retention time as the didansyl derivative of the standard (Fig. 1b). Co-elution of the urine sample and standard GH yields a double peak in which the didansylated portion is intensified (Fig. 1c).

This portion of the peak, ascribed to the didansylated derivative of GH, was collected and rechromatographed on the analytical column. As shown in Fig. 2b, the identification of this peak as that of the didansylated standard GH (Fig. 2a) was confirmed. Fig. 2c shows the chromatographic profile obtained by co-eluting the standard and the isolated compound.

In order to prove unambiguously the chemical nature of the compound, further analyses were performed. First, the fraction eluted from the preparative column (Fig. 1b) was submitted to acid hydrolysis, to split the glycosidic bond and to release hydroxylysine. This treatment resulted in a peak with the same retention time as that of the didansyl derivative of a standard of hydroxylysine (see Fig. 2d and e). Fig. 2f shows the chromatographic profile obtained by co-eluting the standard and the isolated compound.

To obtain further proof that the product separated by the preparative column is the didansyl derivative of GH, an MS investigation was undertaken. FAB [6] of the glycerol solution of the sample leads to a complex mass spectrum, with peaks at every mass value up to 1200. However, a clear peak corresponding to the $(MH)^+$ species of $(Dns)_2$-GH appears at $m/z$ 791, with a signal-to-noise ratio of ca. 10:1 (Fig. 3).

The complexity of the FAB spectrum did not allow any investigation of the

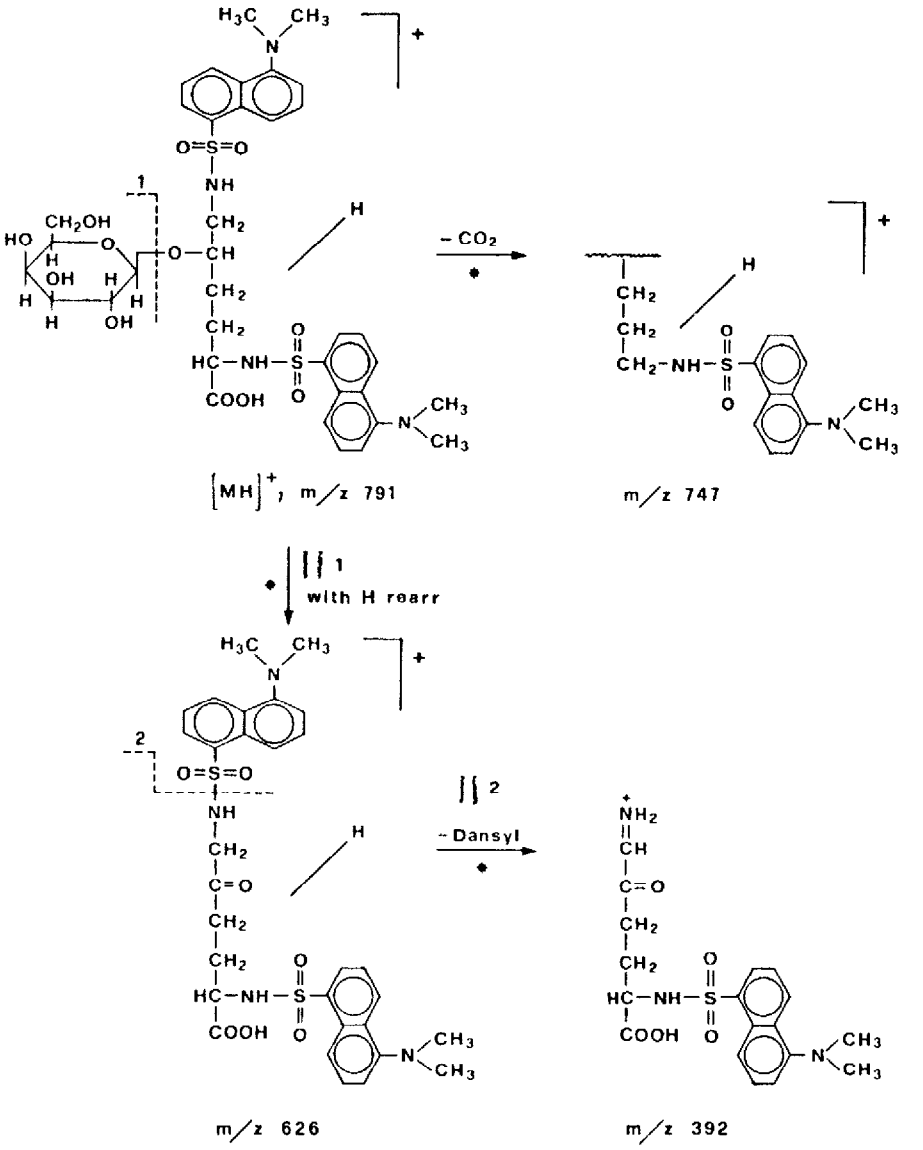


Fig. 5 FAB-induced fragmentation of didansyl GH, as determined from metastable ion data.

possible fragment ions, which could be highly diagnostic from the structural point of view. Thus we undertook a series of experiments on the naturally occurring decompositions in the first field-free region of the apparatus [10], by means of $B/E$=constant linked scans [9]. By such an approach the unambiguous identification of the fragment ions of a preselected species is easily

achieved, with the complete suppression of the 'chemical noise' present in the FAB mass spectrum.

The *B/E* linked scan of ions at *m/z* 791 (Fig. 4) shows the formation of abundant ionic species at *m/z* 626 and, to a lesser extent, of fragments at *m/z* 747. While the former can be easily explained by cleavage of the ether bond between the sugar and the amino acidic moieties (cleavage 1 in Fig. 5), the latter (corresponding to the loss of 44 mass units) can be explained by a decarboxylation reaction. Furthermore, *B/E* linked scans performed on ionic species at *m/z* 626 show the formation of an ion at *m/z* 392, corresponding to the loss of a dansyl moiety (cleavage 2, Fig. 5).

In conclusion, FAB MS, linked with metastable data studies, has led to an unequivocal structural identification of $(Dns)_2$-GH.

The method described in this paper to prepare the pure didansyl derivative of GH could be easily reproduced in any laboratory that currently uses HPLC to determine the amounts of GH excreted in urine.

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