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Short communication

Assay for 5-hydroxylysine and L-lysine in human and rat urine and in bone by gas chromatography

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Abstract

An accurate method for the determination of collagen to study its distribution and turn-over in different tissues is described. 5-Hydroxylysine (5Hylys) is an amino acid that is apparently present in no other protein except collagen and, as it is metabolised only to a minor degree compared with 4-hydroxyproline (4Hypro), it has been suggested as a better marker of the collagen metabolism. Interest in this amino acid has increased recently because the levels of 5Hylys in urine and in different tissues may offer a new basis for detecting pathologies of the collagen molecule. This paper describes a method for the quantitative determination of 5Hylys and lysine (Lys) by gas chromatography (GC) in human and rat urine and in rat bone. The limit of detection was 350 pmol ml^{-1} for 5Hylys and 200 pmol ml^{-1} for Lys for all the biological samples. This method therefore provides a complete view of the metabolism of this amino acid and of the tissue it comes from. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lysine; 5-Hydroxylysine

1. Introduction

Various reports describe the chromatographic [1–4] and HPLC techniques [5–7] for the analysis of free amino acid or total amino acid after hydrolysis of the proteins. Methods are available for measuring all the amino acids of a protein although sometimes only one amino acid needs to be quantified, as in the case of the collagen molecule.

Several urinary metabolites are commonly measured to evaluate collagen turnover. The most widely used is 4Hypro [8] but this does not provide quantitative information on collagen breakdown for different reasons. Firstly, 4Hypro is a characteristic but not exclusive amino acid of collagen and its urinary excretion is affected by diet [9]. Moreover, only 20% of 4Hypro is excreted in the urine because most is transaminated by liver enzymes [10,11].

Another marker has therefore been considered: 5Hylys is an exclusive amino acid of collagen and, as it is metabolised only to a small degree, its urinary excretion reflects true collagen breakdown [12–14]. Moreover, it is not affected by diet [15]. Excretion of free 4Hypro and 5Hylys is very limited; 4Hypro is excreted predominantly as a peptide, while 5Hylys is excreted mainly as a glycoside, glucosyl-galactosyl-hydroxylysine (glc-gal-Hylys) or galactosyl-hydroxylysine (gal-Hylys) [12–18].

A complicated method for measuring 5Hylys by thin-layer chromatography was described by

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Blumenkrantz and Prockop [19] and was applied to proteic hydrolysate but not to collagen. This paper describes a new gas chromatographic (GC) method for the evaluation of total 5Hylys in urine and different tissues, to assess different degrees of pathology. This sensitive GC method permits the evaluation of 5Hylys from both isomers (δ-Hylys and allo-δ-Hylys, obtained by acid hydrolysis) and of Lys after ion-exchange chromatography, derivatization and quantitative determination by fused-silica capillary gas chromatography.

2. Experimental

2.1. Reagents and chemicals

DL+allo-δ-hydroxylysine·HCl and L-lysine were supplied by Calbiochem (San Diego, CA, USA). The external standard, *n*-hexadecane, supplied by Carlo Erba (Milan, Italy), was used at a concentration of 5 nmol ml^{-1} in ethyl acetate, also supplied by Carlo Erba.

Acetic acid was supplied by Merck (Darmstadt, Germany); *n*-propanol and acetyl chloride were purchased from Farmitalia-Carlo Erba (Milan, Italy). Pure trifluoroacetic anhydride was purchased from Fluka (Buchs, Switzerland). Columns (2.5×1 cm I.D.) packed with Bio-Rex 70 (100–200 mesh, NH_4^+) were purchased from Bio-Rad Laboratories (Richmond, CA, USA).

2.2. Apparatus

GC analysis was done on a Dani 6500 instrument (Dani, Monza, Italy) with a capillary programmed temperature vaporizer (PTV) injector equipped with a flame ionization detector (FID), a Shimadzu C-R3A Chromatopac integrator and a wall-coated open tubular (WCOT) fused-silica capillary column (30 m×0.33 mm I.D.) coated with SPB_5, film thickness 1 μm (Supelco, Milan, Italy). The GC injector temperature was increased from 60 to 300°C and the detector temperature was 300°C. The split was opened 42 s after injection of the total sample into the PTV injector and the temperature programme was started. The column temperature was programmed at 130°C and was held for 5 min then raised to 200°C at 5°C min^{-1}. This temperature was held for 10 min, in order to remove the contaminants from the column. The carrier flow-rate was 6 ml min^{-1} (hydrogen through the capillary column).

The mass spectrometric (MS) assay was carried out on a VG TS-250 (Fisons Instruments, Manchester, UK) interfaced with a SPB_5 capillary column (30 m×0.33 mm I.D).

2.3. Biological samples

Human and rat urine were collected according to standard procedures. Rat bone (femur and humerus) was disarticulated and soft tissues were carefully removed.

Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., Suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

2.4. Procedures

2.4.1. Urine

One ml of urine was hydrolysed in an equal volume of 37% HCl in a sealed glass vial at 110°C for 72 h; 0.1 ml of hydrolysate was evaporated to dryness on a Rotavapor under vacuum at 50°C.

2.4.2. Bone

Bone was pounded in a steel mortar and hydrolysed with 6 *M* HCl in a tightly capped vial at 110°C for 72 h. An amount of hydrolysate equivalent to 2 mg of fresh tissue was evaporated to dryness on the Rotavapor under vacuum at 50°C. The sample was dissolved in bidistilled water and applied to a column (2.5×1 cm I. D.) packed with Bio-Rex 70 (100–200 mesh, NH_4^+). After washing with 75 ml of bidistilled water, the sample was eluted with 10 ml of acetic

acid 2 *M* and then dried on the Rotavapor under vacuum at 45°C.

2.5. Derivatization

The carboxy group of 5Hylys and Lys was esterified with 3 ml of 10% dry acetyl chloride in *n*-propanol. The white residue that appears after addition of the derivatization mixture does not interfere with the subsequent reactions because there is an excess of acetyl chloride. Each glass vial was sealed, mixed and left to react overnight at 80°C in a Reacti-Therm heating module (Pierce, Rockford, IL, USA). The glass vial was left to cool, then centrifuged at 2000 *g* for 5 min; 2.5 ml of the supernatant was dried under nitrogen flow and 400 μl of trifluoroacetic anhydride were added. The vial was then sealed and allowed to react for 1 h at 80°C. The samples were cooled and dried under nitrogen flow. A suitable volume of the external standard solution containing *n*-hexadecane (5 nmol ml^{-1}) in ethyl acetate was added to the sample and 1 μl was injected with a Hamilton syringe in the gas chromatograph. *n*-Hexadecane has different physiochemical properties from our analytes and is added only after sample preparation because its only purpose is to correct the variation in the quantity injected, to control the efficiency of the detector, the reproducibility of the retention time and to allow the identification and the quantitative determination of the analytes.

This esterification step was already used for the esterification of other amino acid such as 3-methylhistidine [20] and it worked out fine for Hylys. Other methods such as a single step silylation were used to obtain *tert*-butyldimethylsilyl derivatives [21] but did not give satisfactory results.

2.6. Preparation of the calibration curve

To check the linearity of the method, we analyzed final concentrations of 350, 700, 1400, 2800, 3200 pmol ml^{-1} of 5Hylys and 200, 400, 800, 1600, 2400 pmol ml^{-1} of Lys. The standard samples were dried on a Rotavapor under vacuum at 45°C and derivatized as described above. A 1 ml volume of *n*-hexadecane (5 nmol ml^{-1}) was then added to each dried standard sample and 1 μl of each sample was analysed repeatedly (at least in quadruplicate) by GC.

2.7. Analytical recovery

To test the recovery of 5Hylys and Lys from the Bio-Rex 70 columns we supplemented five previously analyzed samples of human and rat urine and rat bone with 5Hylys to final concentrations of 350, 700, 1400 pmol ml^{-1} and with Lys to 200, 400, 800 pmol ml^{-1}.

Two assays were done to test the hydrolysis conditions. For the first assay, ten 1 ml samples from a pool of human urine were hydrolysed in hydrochloric acid for 72 h at 110°C, then evaporated to dryness under vacuum. To five dried samples 1 ml of the same pool of human urine was added and hydrolysed once more for 72 h at 110°C. For the second assay, six 1 ml samples from a pool of human urine were supplemented with 5Hylys to final concentrations of 350, 700, 1400 pmol ml^{-1} and with Lys to 200, 400, 800 pmol ml^{-1}, then hydrolysed in hydrochloric acid for 72 h at 110°C.

The same assays were done with a pool of rat urine and bone.

2.8. Stability of biological samples on storage

2.8.1. Urine and bone

The samples were stored at −20°C and their stability was checked every week for 2 months.

2.8.2. Hydrolyzed samples

Hydrolyzed samples were stored at +4°C and their stability was checked every 2 weeks for 4 months.

2.8.2. Derivatized samples

After addition of the standard solution containing *n*-hexadecane, derivatized samples were stored at +4°C and their stability was checked every day for 1 week.

All the samples were stored in glass tubes and the stability experiments were run in quadruplicate.

3. Results and discussion

Fig. 1a–d show typical chromatograms of standards, rat and human urine and rat bone samples.

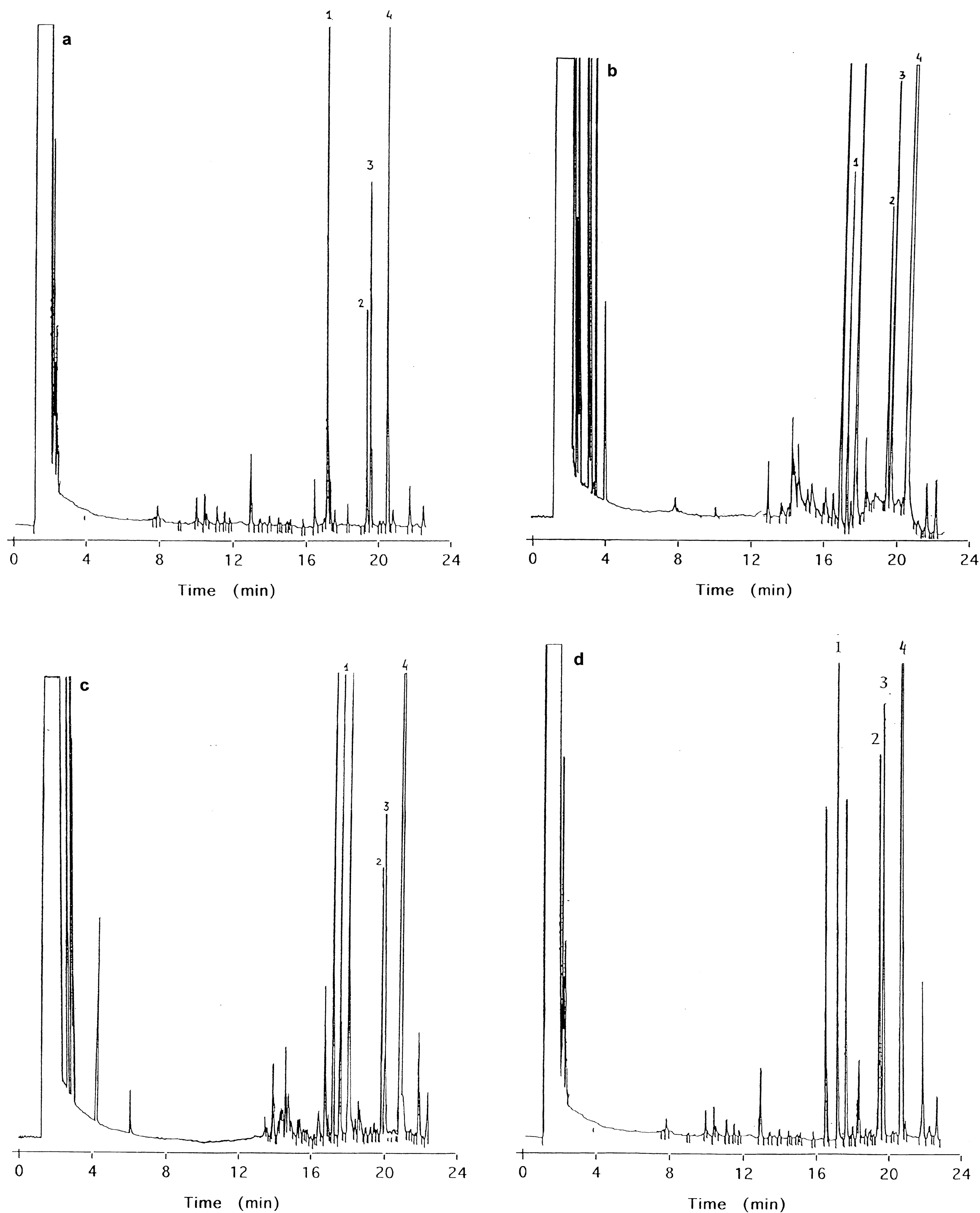


Fig. 1. (a) Chromatograms of standard solution with internal standard *n*-hexadecane (5 nmol ml^{-1}) (1), δ-Hylys (1.3 nmol ml^{-1}) (2), allo-δ-Hylys (1.8 nmol ml^{-1}) (3), and Lys (3.5 nmol ml^{-1}) (4). (b) Chromatograms of rat urine with internal standard (1), δ-Hylys (2), allo-δ-Hylys (3), and Lys (4). (c) Chromatograms of human urine with internal standard (1), δ-Hylys (2), allo-δ-Hylys (3), and Lys (4). (d) Chromatograms of rat bone with internal standard (1), δ-Hylys (2), allo-δ-Hylys (3), and Lys (4).

The peaks of the two 5Hylys isomers were identified using the percentage declared by Calbiochem and similar to the ratio between the two peaks. Regardless of the species and biological matrix, the chromatographic profiles were very similar.

The calibration curve for 5Hylys derivative concentrations from 350 to 3200 pmol ml^{-1} and for Lys from 200 to 2400 pmol ml^{-1} indicated good linearity of response within this range. Calculation of the least-squares regression ($y=1.18x-0.03$ for δ-Hylys, $y=1.93x-0.07$ for allo-δ-Hylys and $y=3.43x-0.07$ for Lys) gave a correlation coefficient of 0.999. The lower limit of quantification (LOQ) of the assay was 350 pmol ml^{-1} for 5Hylys and 200 pmol ml^{-1} for Lys.

Recoveries for 5Hylys and Lys in all biological samples from Bio-Rex 70 columns were respectively 99±1 and 98±1 (mean±S.D. of five determinations). Recovery was similar for all the concentrations (data not shown).

The stability studies for all biological and hydrolysed samples and derivatized samples after addition of the standard solution, showed that they contained 100±5% of the initial amount of both amino acid (data not shown).

5Hylys is found in tissues as a glycoside and is excreted manly as glc-gal-Hylys and gal-Hylys. Preliminary hydrolysis is necessary for evaluation of the total amino acid. Many different hydrolysis conditions have been employed. We consider the hydrolysis step very important and therefore established the optimal time and temperature using standards of collagen and urine. We found that 72 h at 110°C were the best conditions to ensure good equilibrium between the two isomers (Fig. 2). These conditions are the only ones in which we obtain the closest quantitation of the two isomers (61% allo- and 39% DL-5-hydroxylysine) according to the Calbiochem batch of our standard (approximately 58% allo- and 42% DL-5-hydroxylysine). Moreover,

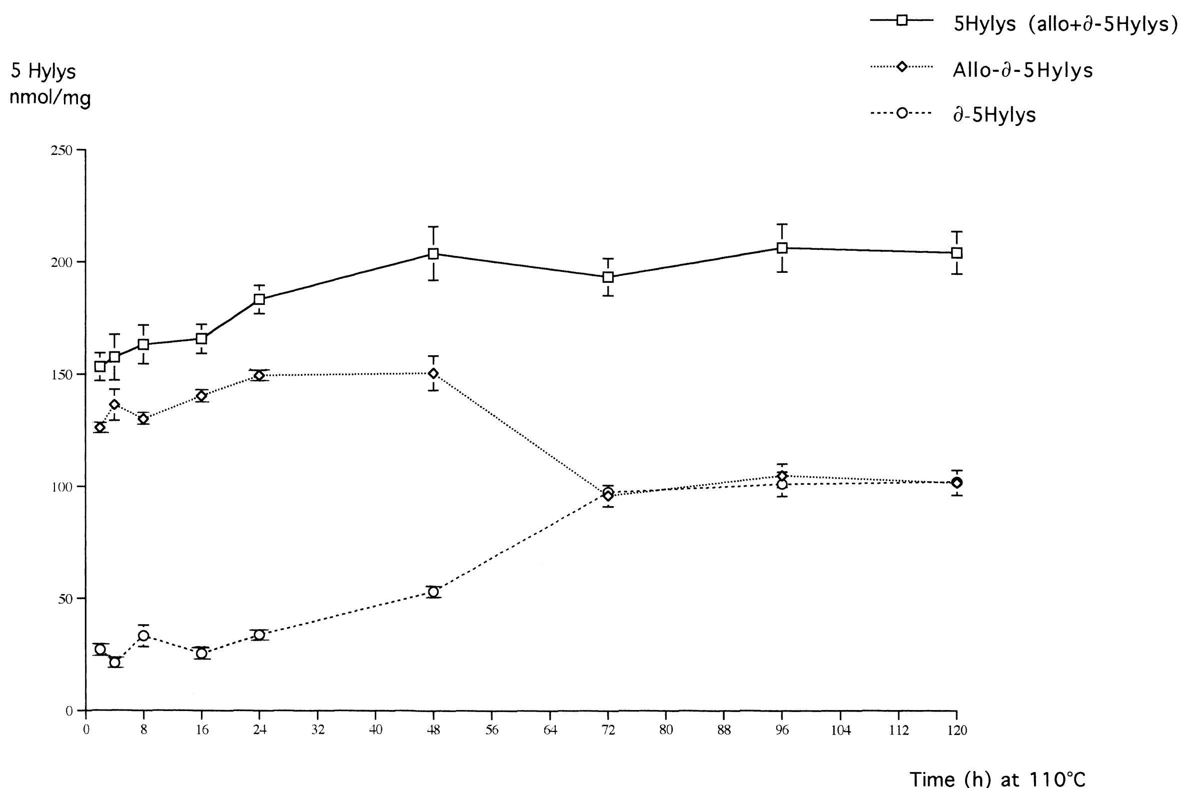


Fig. 2. Hydrolysis conditions for biological samples, showing the hydrolysis of rat urine.

these are the conditions which result in complete hydrolysis of collagen in the biological samples.

The first assay of the recovery of hydrolysis showed that the five hydrolysed samples to which 1 ml of the same pool of urine was added and hydrolysed once more under the same conditions, had double the amount of 5Hylys and Lys of the samples hydrolysed only once (data not shown). Recovery from hydrolysis in which 5Hylys and Lys were added was 95–96% (data not shown).

The precision and accuracy of the 5Hylys and Lys assay in all biological samples was evaluated on the basis of the within- and between-assay precision. 5Hylys and Lys within-assay precision was determined by testing five replicates of each biological sample. The between-run precision was evaluated from the analysis of the same samples in three different experiments. The accuracy and the within- and between-run variation were less than 10% for high and low concentrations for both amino acids (Table 1).

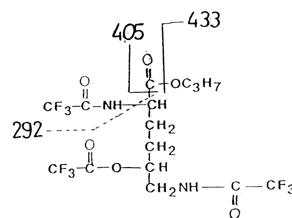
The synthesis of the *n*-propyltrifluoroacetyl derivative was confirmed by GC–MS with standard samples of 5Hylys and Lys (Figs. 3 and 4). The mass spectrum indicates that all reactive sites were blocked by the trifluoroacetyl group. The mass spectra of the two isomers of 5Hylys are identical. Chromatographic peaks of biological samples were identified with good precision by comparison of the mass spectra of the biological compound and of authentic reference standards.

Table 2 reports assays of 5Hylys in human and rat urine, Table 3 in rat bone.

In conclusion, this GC method could be a useful tool for investigating the role of collagen in inflammatory disorders such as septic shock, cystic

Table 1
Within-assay ($n=5$) and between-assay ($n=15$) variation in human and rat urine and in rat bone

Biological sample	Coefficient of variation (%)			
	Within-assay variation		Between-assay variation	
	5HyLys	Lys	5HyLys	Lys
Human urine	7.42	5.92	5.73	4.74
Rat urine	6.35	6.84	4.83	5.19
Bone rat	8.01	7.12	7.09	5.82



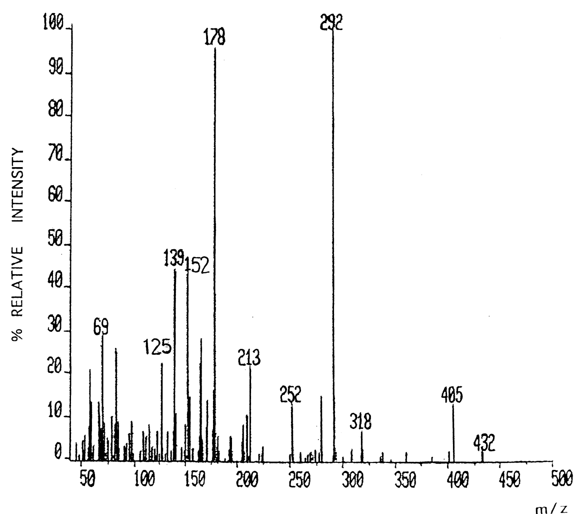


Fig. 3. Mass fragmentation pattern of the 5Hylys (allo-δ and δ-Hylys) derivative.

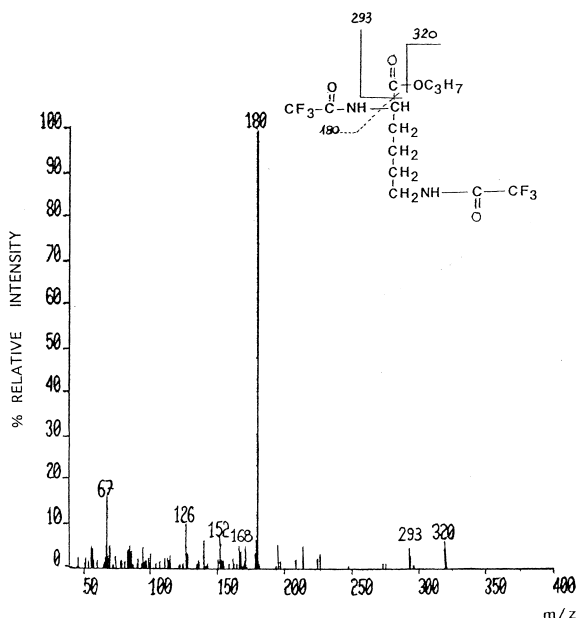


Fig. 4. Mass fragmentation pattern of the Lys derivative.

Table 2
Assays of 5Hylys in human urine and rat urine

	Human urine	Rat urine
Creatinine (CN) (mmol/24 h)	13.60±1.87	0.07±0.01
allo-δ-Hylys/CN (μmol mmol^{-1})	3.63±0.14	22.82±1.46
δ-Hylys/CN (μmol mmol^{-1})	3.75±0.21	18.73±1.25
5Hylys (allo-δ+δ-Hylys)/CN (μmol mmol^{-1})	7.83±0.33	41.69±2.72
L-lys/CN (μmol mmol^{-1})	42.65±3.74	209.43±11.42
allo-δ-Hylys (μmol/24 h)	49.30±6.90	1.62±0.16
δ-Hylys (μmol/24 h)	51.08±9.37	1.33±0.15
5Hylys (allo-δ+δ-Hylys) (μmol/24 h)	100.41±15.84	2.96±0.29
L-lys (μmol/24 h)	580.07±93.03	14.87±2.50

Table 3
Assays of 5Hylys in rat bone

	Bone from adult female rats	
	Femur	Humerus
allo-δ-Hylys (mmol mg^{-1})	6.78±0.55	7.77±0.86
δ-Hylys (mmol mg^{-1})	6.29±0.68	7.71±0.69
5Hylys (allo-δ+δ-5Hylys) (mmol mg^{-1})	13.07±1.23	15.48±1.55
L-lys (mmol mg^{-1})	83.63±6.73	91.17±7.74

fibrosis, arthrosis and rheumatoid arthritis, all disorders associated with an excess of collagen in urine and in tissue. An accurate method of measuring bone turnover is also needed in clinical investigations of osteoporosis.

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