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High-performance liquid chromatographic method for measuring hydroxylysine glycosides and their ratio in urine as a possible marker of human bone collagen breakdown

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ABSTRACT

Glucosyl-galactosyl-hydroxylysine (GGHYL) and galactosyl-hydroxylysine (GHYL) are constituents of collagen protein. The ratio of the two hydroxylysine glycosides varies with the collagen type and, moreover, for a given collagen type, it also varies according to the connective tissue. For example, in type I collagen (the most abundant in the body), the GGHYL/GHYL ratio tends to be greater in soft connective tissues and lower in bone. The hydroxylysine glycosides are not recycled during collagen turnover and are excreted in the urine. Therefore, the urinary GGHYL/GHYL ratio, which reflects the proportion of the two metabolites in the various collagens, may indicate the type of connective tissue affected by pathological turnover, and may thus be a promising marker of bone metabolism. In this paper a method is described for the measurement of urinary hydroxylysine glycosides by reversed-phase liquid chromatography after purification of the sample by solid-phase extraction. The method presented is analytically reliable and suitable for routine use in a clinical laboratory.

INTRODUCTION

The products of bone collagen breakdown found in the urine are current candidates for biochemical, non-invasive markers of alterations in bone metabolism [1]. In addition to the measurement of hydroxyproline, lysylpyridinoline and hydroxylysylpyridinoline in urine, galactosyl-hydroxylysine (GHYL), a hydroxylysine glycoside, has recently been suggested to be of clinical value because bone collagen breakdown can be considered its main source [2].

The glycosylation of hydroxylysine residues is a modification that occurs in all known collagens, although to different degrees. Hydroxylysine undergoes mono- and diglycosylation, producing GHYL and glucosyl-galactosyl-hydroxylysine (GGHYL), respectively [3–8]. The glycosylation is more complete in soft connective tissues, whereas in bone the phenomenon is less marked. In skin collagen the GGHYL/GHYL ratio is *ca.* 1.6:1 and in bone collagen it is 1:7 [9,10].

The clinical significance of the GHYL measurement is questionable when a high turnover of soft connective tissue is present, and also if a specific α-glucosidase is present and active in human kidney [11]. Its clinical significance might be improved by the simultaneous measurement of excreted GGHYL and GHYL [12]. Owing to the different GGHYL/GHYL ratios for collagen in skin and in bone, the urinary GGHYL/GHYL

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ratio becomes higher in disorders of the skin and lower in pathological conditions involving bone collagen [9]. In addition, the measurement of the GGHYL/GHYL ratio offers a distinct advantage over GHYL alone because the ratio is independent of renal function, and recovery is the same for both analytes, so no internal standard is necessary.

Several excellent methods for the measurement of the GGHYL/GHYL ratio have been published, all of which use ion-exchange chromatographic separation followed by colorimetric quantitation after reaction with ninhydrin [13–15]. This paper describes a simple and reliable method for the measurement of both glycosides, GGHYL and GHYL, by reversed-phase chromatography using precolumn derivatization with 4-dimethylaminoazobenzene-4-sulphonyl chloride (dabsyl chloride) and spectrophotometric detection. The method may be a suitable alternative to conventional amino acid analysis, especially when suitable equipment for the latter is not available.

EXPERIMENTAL

Apparatus and columns

An HPLC system with spectrophotometric detection at 436 nm was used. The system included a Model 360 autosampler, two Model 340 pumps, a UV–VIS Model 432 spectrophotometer and an MT2 system of computerized management, all manufactured by Kontron (Milan, Italy). A C_{18} ODS2 Spherisorb column (15 cm × 0.46 cm I.D., 3 μm; Phase Separations, Deeside Clwyd, UK) and a C_{18} ODS2 Spherisorb precolumn (5 cm × 0.46 cm I.D., 3 μm) were used.

Mobile phases

A 50 m*M* acetate buffer (pH 5.08) was prepared by dissolving 8.2 g of anhydrous sodium acetate (Fluka BioChemika, Buchs, Switzerland) in *ca.* 1900 ml of deionized water (Milli Q-Plus, Millipore, Bedford, MA, USA). The pH was adjusted to 5.08 with 37% HCl (Baker, Deventer, Netherlands). The solution was diluted to a volume of 2 l and filtered through a Millipore 0.2-μm filter directly into a sterile glass bottle, and the pH was rechecked. The buffer (buffer A) prepared in this way was stable for one week at room temperature. The pH was checked before use.

Solvent B was HPLC-grade acetonitrile (Baker).

Reagents and standards

HPLC-grade methanol was from Baker, and deionized water from Millipore.

Sodium hydrogencarbonate buffer ($NaHCO_3$) (200 m*M*, pH 10.00) containing 470 m*M* lithium chloride (LiCl) was prepared by dissolving 16.8 g of $NaHCO_3$ (Merck, Darmstadt, Germany) and 20 g of LiCl (Merck) in 900 ml of deionized water, and adjusting the pH to 10.00 with 10 *M* NaOH (Merck). The solution was brought up to a volume of 1000 ml and filtered through a 0.45-μm filter (Millipore), and the pH was rechecked.

Sodium hydrogencarbonate buffer (200 m*M*, pH 9.20) was prepared by dissolving 16.8 g of $NaHCO_3$ (Merck) in 900 ml of deionized water (Millipore), and adjusting the pH to 9.20 ± 0.05 with 10 *M* NaOH. The volume brought up to 1000 ml. The solution was filtered through a 0.45-μm filter (Millipore) and the pH checked.

A 66-mg amount of dabsyl chloride (Fluka), purified according to Chang *et al.* [16], was dissolved in 10 ml of HPLC-grade acetone (Merck) in a polyethylene test-tube. The tube was capped, inverted and shaken gently for at least 15 min to dissolve the dabsyl chloride completely.

Ethanol–water (70:30) solution was obtained by mixing 70 ml of ethanol (Merck) with 30 ml of prefiltered deionized water (0.2 μm, Millipore).

A stock solution of hydroxylysine (1 m*M*) was prepared by dissolving 198.6 mg of hydroxylysine hydrochloride (Fluka) in 1000 ml of deionized water. Working solutions with concentrations of 5, 10, 20, 50 and 100 μmol/l were obtained by diluting the stock solution. Aliquots of the stock solutions were kept at −80°C.

GGHYL and GHYL, obtained by hydrolysis of marine sponges [17], were kindly supplied by Professor R. Tenni (Department of Biochemis-

try, University of Pavia, Pavia, Italy). Two working solutions were prepared of GGHYL (2880 μM) and GHYL (1145 μM). These concentrations were calculated on the basis of a primary hydroxylysine standard, assuming that hydroxylysine and its glycosides react with dabsyl chloride with the same stoichiometry (didabsylation) and all derivatives have the same molar extinction coefficient.

Sample preparation

The urine samples used were taken from the second urine of the day collected under fasting conditions according to the method of Nordin. Immediately after collection the samples were analysed to determine the creatinine concentration (alkaline picrate, BBI, Milan, Italy) and were then stored in aliquots at −20°C until use.

Aliquots were thawed, mixed gently and centrifuged (2500 *g*) for 10 min and 0.5 ml of the supernatant was diluted with 5 ml of deionized water in a 10-ml test-tube. The resulting solution was mixed carefully, and the pH checked and adjusted when necessary to 5.30 ± 0.10 with 0.05 m*M* HCl or NaOH. The extraction columns (Bakerbond sulphonic acid, 500 mg, Baker) were conditioned sequentially with 3 ml of methanol (Baker) and 6 ml of deionized water (Millipore). The diluted sample (5 ml) was injected and the column was washed with 3 ml of methanol and 6 ml of deionized water. The glycosides were eluted with 1.5 ml of a buffer solution consisting of 200 m*M* sodium hydrogencarbonate and 470 m*M* LiCl (pH 10.00) in a glass test-tube with PTFE screw top (12 mm × 75 mm) containing 130 μl of 2 *M* NaOH.

The eluate was dried by vacuum centrifugation (Savant 290, Farmingdale, NY, USA) for 2 h at 40°C, and the dry residue was reconstituted with 1 ml of 200 m*M* sodium hydrogencarbonate buffer (pH 9.20). To ensure complete solution, the test-tube was placed in an ultrasonic bath for 2 min. After checking the pH of the sample and adjusting if necessary (acceptable range 9.15–9.35), 1 ml of 20 m*M* dabsyl chloride solution in acetone was added. The sample was then vortex-mixed and incubated at 70°C for 15 min. The sample was allowed to cool to room temperature, 1 ml of ethanol–water (70:30) was added, and the sample was vortex-mixed and centrifuged (2500 *g*) for 5 min. The supernatant was transferred to an autosampler vial.

For chromatographic analysis, 20 μl of the derivatized sample were injected into the column. The chromatographic separation of glycosides was achieved in gradient mode (Table I) at a flow-rate of 0.8 ml/min. The column temperature was 25°C, and the detection wavelength was 436 nm (0.02 a.u.f.s.). The areas of the peaks were calculated.

Evaluation of analytical procedure

Calibration was performed with aqueous hydroxylysine solutions of 5, 10, 20, 50 and 100 μM. The calibration curve was obtained by plotting the areas of the peaks against the hydroxylysine concentrations of the standards.

For determination of retention times, aqueous solutions of the glycosides [GGHYL and GHYL, not submitted to solid-phase extraction (SPE)] were processed before each analytical series.

The analytical precision of the method within and between batches was evaluated using three urine samples at different concentrations of GGHYL (7.6, 13.9, 34.3 μmol/l) and GHYL (5.4, 11.8, 35.6 μmol/l), five times on three different days.

TABLE I

GRADIENT ELUTION PROFILE FOR THE SEPARATION OF GGHYL AND GHYL

Time (min)	Buffer A (%)	Solvent B (%)
0	80	20
0.01	Inject sample	
0–35	54.5	45.5
35–42	53.4	46.6
42–43	10	90
43–48	10	90
48–49	80	20
49–60 (column equilibration)	80	20

To evaluate the total recovery (SPE plus derivatization), 10 μl of the working solutions of GGHYL and GHYL were added to 0.5 ml of urinary samples at different concentrations of the two analytes. The volume was then brought to 5.5 ml with deionized water, and 5 ml of the solution were injected into the SPE column and processed as described above. The recovery test was performed in duplicate.

To check the recovery in the derivatization phase, 5 ml of deionized water were injected into the SPE column, and 10 μl each of the GGHYL and GHYL working solutions were added to the eluate. The samples were then dried and derivatized. The test was performed in duplicate.

To determine the total linearity (SPE plus derivatization), increasing amounts of GGHYL and GHYL were added to a urine sample. The samples thus obtained were processed as described above. To determine the partial linearity (derivatization alone), graded dilutions of the working GGHYL and GHYL solutions were made with deionized water, and the samples obtained were dried and submitted to derivatization without SPE. All tests were performed in duplicate.

The limit of detection was considered equal to three standard deviations of the blank background signal (water plus dabsyl chloride).

To evaluate the stability of the products of derivatization, the samples were placed in airtight vials and analysed by HPLC at time zero and after 24 h at room temperature. The test was performed in duplicate for ten samples.

To determine the reference interval of GGHYL and GHYL in the second urine sample of the day collected under fasting conditions, these analytes were measured in fifteen fertile women in good health aged between 25 and 45 years.

RESULTS

Fig. 1 shows a typical chromatogram of an aqueous standard of GGHYL and GHYL, Fig. 2 of a urine sample, Fig. 3 of the same sample with added GGHYL, and Fig. 4 of the same sample

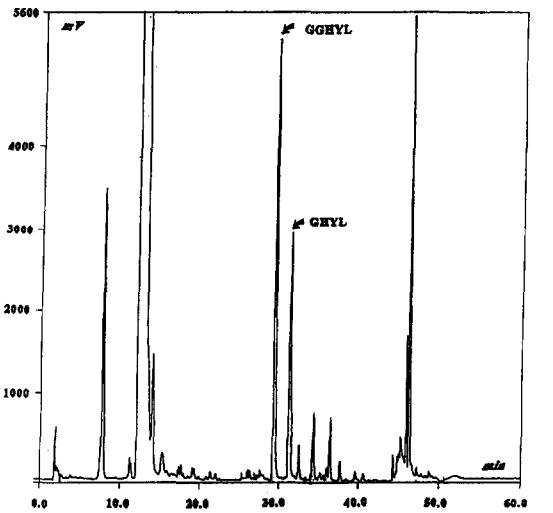

Fig. 1. Chromatogram of an aqueous standard of GGHYL and GHYL.

with added GHYL. The occasional appearance of an asymmetric peak of GGHYL, as in Fig. 3, is probably due to the presence of diastereoisomers of didabsylated glycoside and reflects the normal fluctuation of column efficiency. The same pattern occurs, for the same reasons, with GHYL. The retention times of GGHYL and GHYL were, respectively, 29 ± 0.1 and 31 ± 0.1

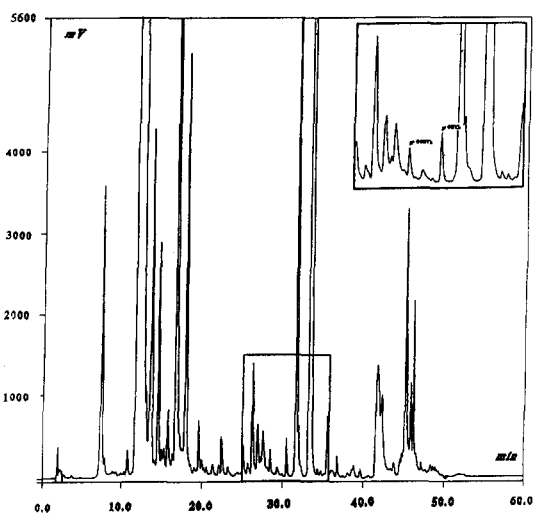

Fig. 2. Chromatogram of a urine sample.

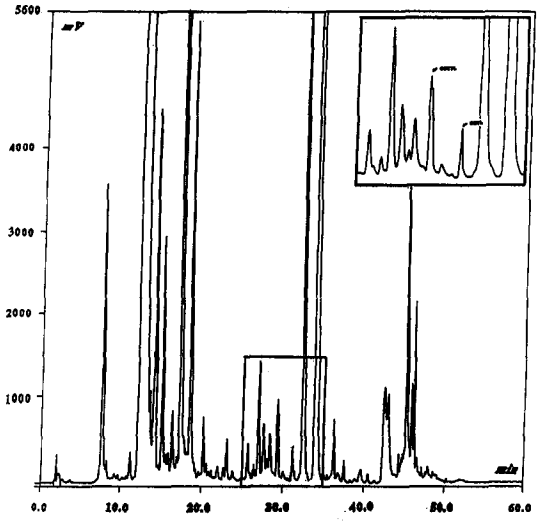

Fig. 3. Chromatogram of the same urine sample of Fig. 2 with added GGHYL.

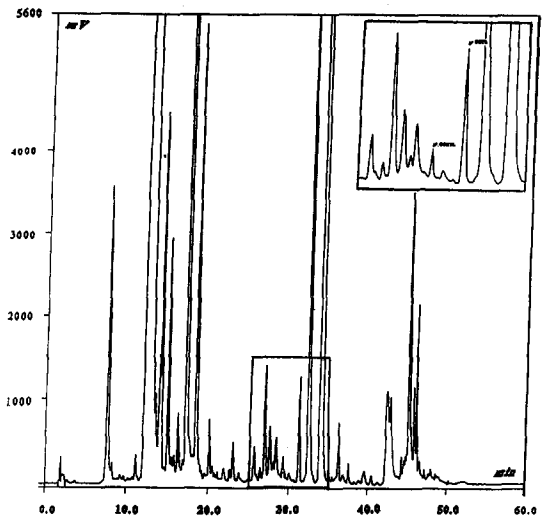

Fig. 4. Chromatogram of the same urine sample of Fig. 2 with added GHYL.

min: the difference was always 2.0 min. When a blank was injected, no interfering peaks appeared in correspondence with the retention times of GGHYL and GHYL.

The findings of the precision test (within- and between-batch) are listed in Table II. The coefficient of variation (C.V.) was generally less than 6% for the entire process: SPE and derivatization.

The results of the recovery tests are given in Table III, and those of the linearity tests in Table IV. The limit of detection for GGHYL and GHYL was 0.5 μmol/l, which corresponds to an injected amount of 8.08 ng of GGHYL and 4.94 ng of GHYL.

The increased stability of the derivatives was demonstrated by the samples submitted to derivatization, maintained at room temperature over-

TABLE II

EVALUATION OF PRECISION

	GHYL (n = 5)		GGHYL (n = 5)	
	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)
Within-batch				
Low level	7.6	6.4	5.2	5.9
Medium level	13.8	4.5	11.6	3.7
High level	34.1	3.2	35.4	2.7
Between-batch				
Low level	7.4	6.6	5.5	6.0
Medium level	14.1	5.7	12.1	5.3
High level	34.5	4.7	35.9	4.0

TABLE III

RESULTS OF THE TESTS OF PARTIAL (DERIVATIZAION ALONE) AND TOTAL (SPE PLUS DERIVATIZATION) RECOVERY

Urine concentration		GGHYL			GHYL		
GGHYL (μM)	GHYL (μM)	Concentration added (μM)	Concentration found (μM)	Recovery (%)	Concentration added (μM)	Concentration found (μM)	Recovery (%)
Total recovery							
9.7	9.5	50.5	43.6	96	19.8	17.8	90
16.9	27.1	50.5	51.3	102	19.8	19.8	100
22.4	35.2	50.5	58.8	94	19.8	16.7	92
Partial recovery							
9.7	9.5	50.5	49.9	99	19.8	19	100
16.9	27.1	50.5	50.5	100	19.8	19.5	98
22.4	35.2	50.5	49.1	97	19.8	19.8	100

night and reinjected the following morning. The peak areas of GGHYL and GHYL varied by only ±1% from those of the previous day.

A range of 1.30–1.60 for the urinary GGHYL/GHYL ratio was found in a reference population of fifteen subjects. In twenty-five women affected by high-turnover osteoporosis, the ratio was between 0.85 and 1.20.

DISCUSSION

We have developed a reliable analytical method with good reproducibility, sensitivity, linearity of response and recovery for the measurement of the concentration of glycosides of hydroxylysine in urine. This method includes a pre-analytical phase (SPE) for purification of the urinary sample and a relatively rapid chromatographic separation (60 min per sample).

Some aspects of the SPE procedure, characterized by strong cation-exchange chromatography with an aromatic sulphonic acid stationary phase, are worthy of further discussion. Before column loading, the pH of the sample must be checked and adjusted, if necessary, to 5.30 ± 0.10. Even if the dilution with water reduces the molarity of the urine, the pH of the final sample is extremely variable owing to the matrix effect of urine (pH and specific weight). As the 200 mM hydrogencarbonate buffer alone is not sufficcient for complete elution of the glycosides from the stationary phase, LiCl (20 g/l) was added to the

TABLE IV

RESULTS OF LINEARITY TESTS

Linearity	Linear regression analysis	
	GGHYL	GHYL
Total	$y = 16.5x - 2.2; r = 0.997$	$y = 5.07x + 6.2; r = 0.996$
Partial	$y = 16.4x - 2.7; r = 0.996$	$y = 6.4x - 0.9\ r = 0.997$

buffer. Li^+ and Na^+ ions exchange with H^+ of the sulphonic radicals, causing a dual effect: a production of CO_2 (from hydrogencarbonate ions) with a consequent slowing down of the elution flow, and a low pH (1.5–2.5) of the eluate. The concentration of the acid eluate by vacuum centrifugation induces a partial hydrolysis of GGHYL to GHYL, because the glycosidic bond between glucose and galactose in diglycoside is more susceptible to acidic conditions than the glycosidic bond between galactose and hydroxylysine present in both GGHYL and GHYL. For these reasons, the eluate was collected into a tube containing 0.13 ml of 2 *M* NaOH.

In the SPE procedure, after adding the sample, a washing step with 3 ml of methanol was performed to facilitate elution of any apolar molecules bound to the aromatic component of the stationary phase.

In the derivatization step, it is important to avoid an incomplete derivatization of the analytes (monodabsylation), which would compromise the accuracy of the analysis: the scarcity of dabsyl chloride in the reaction mixture and the hydrolysis of dabsyl from diderivatizated products during the incubation may both cause an increase in monodabsyl derivatives.

Owing to increased polarity, monodabsyl hydroxylysine and their glycosides are characterized by a reduction of the retention time with respect to the didabsylated forms. In the case of hydroxylysine, the two diastereoisomer mixtures of monoderivatives show retention times of 15 and 18 min, respectively, and the retention time of didabsyl hydroxylysine is 36 min. Fig. 5 shows a chromatogram of a hydroxylysine standard (250 μmol/l) mono- and didabsylated with a dabsyl chloride solution of 0.1 m*M*. The peaks of didabsyl hydroxylysine, as well as those of GGHYL and GHYL, are split, owing to the similar polarity of the diastereoisomers. Our observations are in accordance with others' experiences [18], even though the latter used dansyl chloride. Polarity increases, and therefore retention time decreases, from monodabsyl to didabsyl derivatives and from GGHYL to HYL. Moro *et al.* [19], analysing a dansyl derivative of GHYL in

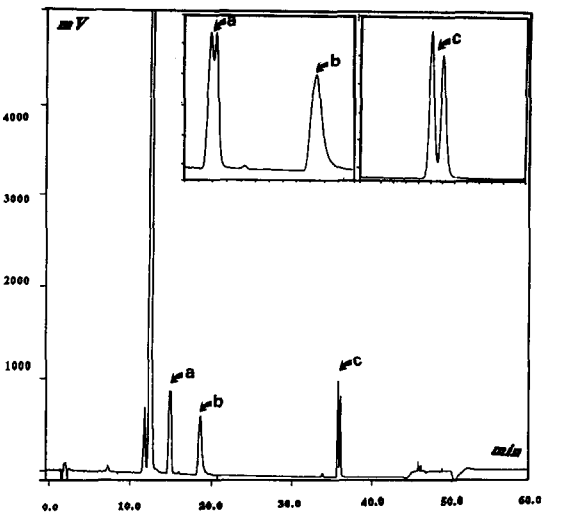

Fig. 5. Chromatogram of an aqueous standard of hydroxylysine: weak derivatization with 0.1 m*M* dabsyl chloride. Peaks: a = monodabsyl hydroxylysine; b = monodabsyl hydroxylysine; c = didabsyl hydroxylysine.

reversed-phase HPLC, reported that GHYL standard appears as a doublet, the first peak being the monodansyl and the second the didansyl derivative. We believe, in accordance with Iwase *et al.* [18], that this discrepancy may arise only from differences in the solvent system used.

To restrict the production of monoderivatives of hydroxylysine, GGHYL and GHYL to less than 5%, the derivatization reaction was carried out with an excess of dabsyl chloride (20 m*M*). After 10 min of incubation at 70°C, the reaction is almost complete; complete derivatization is reached in 15 min but, after 20 min, the recovery of the analytes decreases to 80–90%, probably owing to hydrolysis of dabsyl. The use of dabsyl chloride in the precolumn derivatization reaction represents a good compromise between sensitivity, ease of operation, and cost.

One problem of the high-performance reversed-phase chromatographic procedure was the removal of the ammonia peak from between the peaks of GGHYL and GHYL. This was achieved by increasing the pH of buffer A (50 m*M* acetate buffer) from an initial value of 4.13 (when ammonia elutes just between the glycosides) to 5.08 (where ammonia elution is delayed

without effect on the retention times of GGHYL and GHYL).

Methods for the measurement of the urinary excretion of GGHYL and GHYL should take into account the following considerations. Owing to the different GGHYL/GHYL ratios for collagen in skin and in bone, the ratio of GGHYL and GHYL concentrations in urine was suggested as an index of bone collagen breakdown [12]. It may constitute a marker of bone turnover in addition to urinary excretion of hydroxyproline, lysylpyridinoline, hydroxylysylpyridinoline, and calcium in fasting state. In has indeed been shown that in Paget's disease patients this ratio is lower than normal, and increased after calcitonin and etidronate treatments [12]. It has also been shown that this ratio was always higher than 1.0 in adults and lower in growing infants [8,10,20,21,23,24].

We believe that measurement of the GGHYL/GHYL ratio offers a potential clinical advantage over measurement of GHYL alone, because it reflects the specific collagen preferentially degraded [8], because it is not affected by the glomerular filtration rate [9], and because the value obtained is a ratio, rather than an absolute number, so no internal standard is necessary in the chromatographic separation.

In conclusion, the urinary GGHYL/GHYL ratio can be considered as a promising marker in detecting pathological alterations of bone metabolism. The method presented here allows the ratio to be measured easily and accurately in the routine clinical laboratory. Studies are in progress in this laboratory to assess its clinical value.

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