Description

This invention relates to a method for assaying bone resorption rates. More specifically, it relates to a method for quantitating specific urinary cross-linking amino acids, and peptide fragments that contain those amino acids derived from degraded bone collagen.

Background of the Invention

Osteoporosis is the most common bone disease in man. Primary osteoporosis, with increased susceptibility to fractures, results from a progressive net loss of skeletal bone mass. It is estimated to affect 15-20 million individuals in the United States. Its basis is an age-dependent imbalance in bone remodelling, i.e., in the rates of synthesis and degradation of bone tissue. About 1.2 million osteoporosis-related fractures occur in the elderly each year, including about 538,000 compression fractures of the spine, about 227,000 hip fractures, and a substantial number of early fractured peripheral bones. Twelve to 20% of the hip fractures are fatal because they cause severe trauma and bleeding, and half of the surviving patients require nursing home care. Total costs from osteoporosis-related injuries now amount to at least $7 billion annually (Barnes, O.M., Science, 236, 914 (1987)). Osteoporosis is most common in postmenopausal women who, on average, lose 15% of their bone mass in the 10 years after menopause. This disease also occurs in men as they get older and in young amenorrheic women athletes. Despite the major, and growing, social and economic consequences of osteoporosis, no method is available for measuring bone resorption rates in patients or normal subjects. A major difficulty in monitoring the disease is the lack of a specific assay for measuring bone resorption rates.

Methods for assessing bone mass often rely on measuring whole-body calcium by neutron activation analysis or mineral mass in a given bone by photon absorption techniques. These measurements can give only long-term impressions of whether bone mass is decreasing. Measuring calcium balances by comparing intake with output is tedious, unreliable, and can only indirectly appraise whether bone mineral is being lost over the long term. Other methods currently available for assessing decreased bone mass and altered bone metabolism include quantitative scanning radiometry at selected bone locations (wrist, calcaneus, etc.) and histomorphometry of iliac crest biopsies. The former provides a crude measure of the bone mineral content at a specific site in a single bone. Histomorphometry gives a semi-quantitative assessment of the balance between newly deposited bone seams and resorbing surfaces.

A urinary assay for the whole-body output of degraded bone in 24 hours would be much more useful. Mineral studies (e.g., calcium balance) cannot do this reliably or easily. Since bone resorption involves degradation of the mineral and the organic matrix, a specific biochemical marker for newly degraded bone products in body fluids would be the ideal index. Several potential organic indices have been tested. For example, hydroxyproline, an amino acid largely restricted to collagen, and the principal structural protein in bone and all other connective tissues, is excreted in urine. Its excretion rate is known to be increased in certain conditions, notably Paget's disease, a metabolic bone disorder in which bone turnover is greatly increased. For this reason, urinary hydroxyproline has been used extensively as an amino acid marker for collagen degradation. Singer, F.R., et al. (1978) In: Metabolic Bone Disease, Vol. II (eds. Avioli, L.V. and Krane, S.M.) pp. 489-575, Academic Press, New York.

Goverde (U.S. Patent No. 3,600,132) discloses a process for determination of hydroxyproline in body fluids such as serum, urine, lumbar fluid, and other intercellular fluids in order to monitor deviations in collagen metabolism. In particular, this inventor notes that in pathologic conditions such as Paget's disease, Marfan's syndrome, osteogenesis imperfecta, neoplastic growth in collagen tissues, and in various forms of dwarfism, increased collagen anabolism or catabolism as measured by hydroxyproline content in biological fluids can be determined. This inventor measures hydroxyproline by oxidizing it to a pyrrole compound with hydrogen peroxide and N-chloro-p-toluenesulphonamide followed by colorimetric determination in p-dimethyl-amino-benzaldehyde.

In the case of Paget's disease, the increased urinary hydroxyproline probably comes largely from bone degradation, hydroxyproline, however, generally cannot be used as a specific index. Much of the hydroxyproline in urine may come from new collagen synthesis (considerable amounts of the newly made protein are degraded and excreted without ever becoming incorporated into tissue fabric), and from turnover of certain blood proteins as well as other proteins that contain hydroxyproline. Furthermore, about 80% of the free hydroxyproline derived from protein degradation is metabolized in the liver and never appears in the urine. Kiviriko, K.I. (1970) Int. Rev. Connect. Tissue Res. 5, 93, and Weiss, P.H. and Klein, L. (1969) J. Clin. Invest. 48, 1.

Hydroxylysine and its glycoside derivatives, both peculiar to collagenous proteins, have been considered to be more accurate than hydroxyproline as markers of collagen degradation. However, for the same reasons described above for hydroxyproline, hydroxylysine and its glycosides are probably equally non-specific mark-

ers of bone resorption. Krane, S.M. and Simon, L.S. (1981) Develop. Biochem. 22, 185.

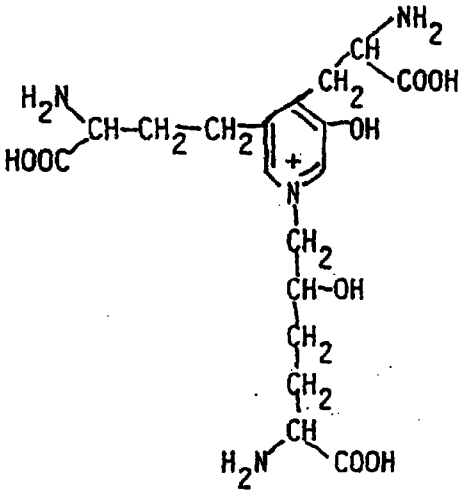
In addition to amino acids unique to collagen, various non-collagenous proteins of bone matrix such as osteocalcin, or their breakdown products, have formed the basis of immunoassays aimed at measuring bone metabolism. Price, P.A. et al. (1980) J. Clin. Invest. 66, 878, and Gundberg, C.M. et al. (1984) Meth. Enzymol. 107, 516. However, it is now clear that bone-derived non-collagenous proteins, though potentially a useful index of bone metabolic activity, are unlikely on their own to provide quantitative measures of bone resorption. The concentration in serum of osteocalcin, for example, fluctuates quite widely both normally and in metabolic bone disease. Its concentration is elevated in states of high skeletal turnover, but it is unclear whether this results from increased synthesis or degradation of bone. Krane, S.M., et al. (1981) Develop. Biochem. 22, 185, Price, P.A. et al. (1980) J. Clin. Invest. 66, 878, and Gundberg, C.M. et al. (1984) Meth. Enzymol. 107, 516.

Collagen Cross-Linking

The polymers of most genetic types of vertebrate collagen require the formation of aldehyde-mediated cross-links for normal function. Collagen aldehydes are derived from a few specific lysine or hydroxylysine side-chains by the action of lysyl oxidase. Various di-, tri-, and tetrafunctional cross-linking amino acids are formed by the spontaneous intra- and intermolecular reactions of these aldehydes within the newly formed collagen polymers; the type of cross-linking residue varies specifically with tissue type (see Eyre, D.R. et al. (1984) Ann. Rev. Biochem. 53: 717-748). Two basic pathways of cross-linking can be differentiated for the banded (67nm repeat) fibrillar collagens, one based on lysine aldehydes, the other on hydroxylysine aldehydes. The lysine aldehyde pathway dominates in adult skin, cornea, sclera, and rat tail tendon and also frequently occurs in other soft connective tissues. The hydroxylysine aldehyde pathway dominates in bone, cartilage, ligament, most tendons, and most internal connective tissues of the body, Eyre, D.R. et al. (1974) vida supra. The operating pathway is governed by whether lysine residues are hydroxylated in the telopeptide sites where aldehyde residues will later be formed by lysyl oxidase (Barnes, M.J. et al. (1974) Biochem. J. 139, 461). The chemical structure(s) of the mature cross-linking amino acids on the lysine aldehyde pathway are unknown, but hydroxypyridinium residues have been identified as mature products on the hydroxylysine aldehyde route. On both pathways and in most tissues, the intermediate, borohydride-reducible cross-linking residues disappear as the newly formed collagen matures, suggesting that they are relatively short-lived intermediates (Bailey, A.J. et al. (1971) FEBS Lett. 16, 86). Exceptions are bone and dentin, where the reducible residues persist in appreciable concentration throughout life, in part apparently because the rapid mineralization of the newly made collagen fibrils inhibits further spontaneous cross-linking interactions (Eyre, D.R. (1981) In: The Chemistry and Biology of Mineralized Connective Tissues (Veis, A. ed.) pp. 51-55, Elsevier, New York, and Walters, C. et al. (1983) Calc. Tiss. Intl. 35: 401-405).

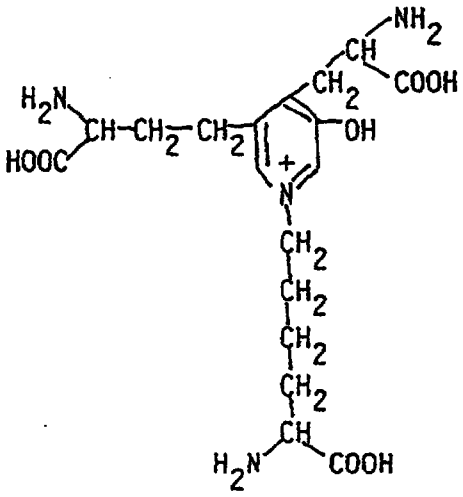
Two chemical forms of 3-hydroxypyridinium cross-link have been identified (Formula I and II). Both compounds are naturally fluorescent, with the same characteristic excitation and emission spectra (Fujimoto, D. et al. (1977) Biochem. Biophys. Res. Commun. 76, 1124, and Eyre, D.R. (1981) Develop. Biochem. 22, 50). These amino acids can be resolved and assayed directly in tissue hydrolysates with good sensitivity using reverse phase HPLC and fluorescence detection. Eyre, D.R. et al. (1984) Analyt. Biochem. 137: 380-388.

FORMULA I



hydroxylysyl pyridinoline (HP)

FORMULA II



lysyl pyridinoline (LP)

In growing animals it has been reported that these mature cross-links may be concentrated more in an unmineralized fraction of bone collagen than in the mineralized collagen (Banes, A.J., et al. (1983) Biochem. Biophys. Res. Commun. 113, 1975). However, other studies on young bovine or adult human bone do not support this concept, Eyre, D.R. (1985) In: The Chemistry and Biology of Mineralized Tissues (Butler, W.T. ed.) p 105, Ebsco Media Inc., Birmingham, Alabama.

The presence of collagen hydroxypyridinium cross-links in human urine was first reported by Gunja-Smith and Boucek (Gunja-Smith, Z. and Boucek, R.J. (1981) Biochem J. 197: 759-762) using lengthy isolation procedures for peptides and conventional amino acid analysis. At that time, they were aware only of the HP form of the cross-link. Robins (Robins, S.P. (1982) Biochem J. 207: 617-620) has reported an enzyme-linked immunoassay to measure HP in urine, having raised polyclonal antibodies to the free amino acid conjugated to bovine serum albumin. This assay is intended to provide an index for monitoring increased joint destruction that occurs with arthritic diseases and is based, according to Robins, on the finding that pyridinoline is much more prevalent in cartilage than in bone collagen. In more recent work involving enzyme-linked immunoassay, Robins reports that lysyl pyridinoline is unreactive toward antiserum to pyridinoline covalently linked to bovine serum albumin (Robins et al. (1986) Ann. Rheum. Diseases 45, 969-973). Robins' urinary index for cartilage destruction is based on the discovery that hydroxylysyl pyridinoline, derived primarily from cartilage, is found in urine at concentrations proportional to the rate of joint cartilage resorption. In principle, this index could be used to measure whole body cartilage loss; however, no information on bone resorption would be available. Indeed, it seems more likely that Robin's assays were largely measuring the increased bone remodelling that occurs in rheumatoid arthritis rather than cartilage destruction.

A need therefore exists for a method that allows the measurement of whole-body bone resorption rates in humans. The most useful such method would be one that could be applied to body fluids, especially urine. The method should be sensitive, i.e., quantifiable down to 1 picomole, and rapidly measure 24-hour bone resorption rates so that the progress of various therapies (e.g., estrogen) can be assessed.

Summary of the Invention

A method for determining the absolute rate of bone resorption comprising quantitating the concentration of peptide fragments having 3-hydroxypyridinium cross-links derived from bone collagen resorption in a body fluid (e.g. urine, synovial fluid, serum) is provided. Thus the invention provides a method of determining the rate of bone resorption, the method comprising quantitating the concentration of a peptide fragment in a body fluid, which is derived from bone collagen resorption and has a 3-hydroxypyridinium cross-link that is lysyl pyridinoline and/or hydroxylysyl pyridinoline. The quantitating steps consists of contacting the body fluid with an immunological binding partner specific to a peptide fragment having 3-hydroxypyridinium crosslinks derived from bone collagen resorption. In one embodiment of the invention, the body fluid is optionally purified prior to the contacting step. This purification step is selected from a number of standard procedures, including cartridge adsorption and elution, molecular sieve chromatography, dialysis, ion exchange, alumina chromatogra-

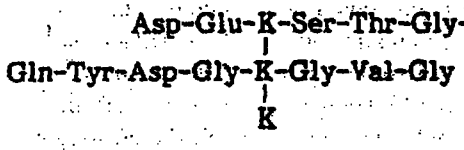
phy, hydroxyapatite chromatography, and combinations thereof.

The invention also encompasses other methods for quantitating the concentration of peptide fragments having 3-hydroxypyridinium cross-links in a body fluid. These methods include electrochemical titration, natural fluorescence spectroscopy, and ultraviolet absorbance. Electrochemical titration may be conducted directly upon a body fluid without further purification. However, when this is not possible due to excessive quantities of contaminating substances, the body fluid is first purified prior to the electrochemical titration step. Suitable methods for purification prior to electrochemical detection include dialysis, ion exchange chromatography, alumina chromatography, molecular sieve chromatography, hydroxyapatite chromatography, and ion exchange absorption and elution.

Fluorometric measurement of a body fluid containing 3-hydroxypyridinium cross-links is an alternative way of quantitating bone resorption. The fluorometric assay can be conducted directly on a body fluid without further purification. However, for certain body fluids, particularly urine, it is preferred that purification of the body fluid be conducted prior to fluorometric assay. This purification step consists of dialyzing an aliquot of urine against an aqueous solution, thereby producing partially purified peptide fragments retained within the nondiffusate. The nondiffusate is then lyophylized, dissolved in an ion pairing solution and absorbed onto an affinity chromatography column. The chromatography column is washed with a volume of ion pairing solution, and, thereafter, the peptide fragments are eluted from the column with an eluting solution.

The invention also relates to peptide fragments derived from bone collagen that contain 3-hydroxypyridinium cross-links; in particular, lysyl pyridinoline cross-links and hydroxylysyl pyridinoline cross-links.

Such fragments have a 3-hydroxypyridinium cross-link derived from the aminoterminal telopeptide domain of bone type I collagen and the following amino acid sequence.

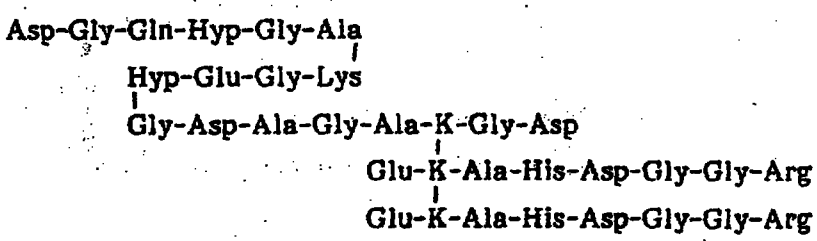


where



is hydroxylysyl pyridinoline or lysyl pyridinoline and,
Gln is glutamine or wholly cyclized pyrrolidone carboxylic acid.

Another fragment containing 3-hydroxypyridinium cross-links is derived from the carboxyterminal telopeptide domain of bone type I collagen and is represented by the formula:



where



is hydroxylysyl or lysyl pyridinoline.

The invention further encompasses a hybridoma which produces monoclonal antibodies specific for the above peptide fragments derived from bone collagen having 3-hydroxypyridinium cross-links. The invention thus provides monoclonal antibodies produced by the hybridoma including those antibodies then coupled to a detectable marker. Examples of detectable markers include enzymes, chromophores, fluorophores, coenzymes, enzyme inhibitors, chemiluminescent materials, paramagnetic metals, spin labels, and radio nucleotides.

The invention also encompasses a test kit useful for quantitating the amount of peptide fragments having 3-hydroxypyridinium cross-links derived from bone collagen resorption found in a body fluid comprising the monoclonal antibody specific for peptide fragments derived from bone collagen and containing 3-hydroxypyridinium cross-links. The monoclonal antibodies of this test kit may be coupled to the detectable markers described above.

Brief Description of the Drawings

FIGURE 1 is a graph of hydroxypyridinium residues in bone collagen versus age.

FIGURE 2 is a graph of the ratio of hydroxylysyl pyridinoline (HP) to lysyl pyridinoline (LP) versus age.

FIGURE 3a is a typical reverse phase HPLC natural fluorescence elution profile of the aminoterminal telopeptides showing the location of the major peptide fragment containing 3-hydroxypyridinium cross-links.

FIGURE 3b is a typical reverse phase HPLC natural fluoresence elution profile of the carboxyterminal telopeptides showing the location of the major peptide fragment containing 3-hydroxypyridinium cross-links.

FIGURE 4A is a typical reverse phase HPLC elution profile of natural fluorescence for a hydrolysate of peptide fragments from normal human urine.

FIGURE 4B is a typical reverse phase HPLC elution profile of natural fluorescence for a hydrolysate of peptide fragments from Paget's disease patent urine.

Detailed Description of the Preferred Embodiments

This invention is based on the discovery that both lysyl pyridinoline (LP) and hydroxylysyl pyridinoline (HP) peptide fragments derived from reabsorbed bone collagen are excreted in the urine without being metabolized. The invention is also based on the discovery that no other connective tissues contain significant levels of LP and that the ratio of HP to LP in mature bone collagen remains relatively constant over a person's lifetime.

FIGURE 1 compares the concentration of HP and LP in both cortical and cancellous human bone with age. It is observed that the concentration of HP plus LP cross-links in bone collagen reaches a maximum by age 10 to 15 years and remains reasonably constant throughout adult life. Furthermore, the ratio of HP to LP, shown in FIGURE 2, shows little change throughout life, remaining constant at about 3.5 to 1. These baseline data demonstrate that the 3-hydroxypyridinium cross-links in bone collagen remain relatively constant and therefore that body fluids derived from bone collagen degradation will contain 3-hydroxypyridinium cross-linked peptide fragments at concentrations proportional to the absolute rate of bone resorption.

Since LP is the 3-hydroxypyridinium cross-link unique to bone collagen, the method for determining the absolute rate of bone resorption, in its simplest form, is based on quantitating the concentration of peptide fragments containing 3-hydroxypyridinium cross-links and preferably lysyl pyridinoline (LP) cross-links in a body fluid. As used in this description and in the appended claims, by quantitating is meant measuring by any suitable means, including but not limited to spectrophotometric, gravimetric, volumetric, coulometric, immunometric, potentiometric, or amperometric means, the concentration of peptide fragments containing 3-hydroxypyridinium cross-links in an aliquot of a body fluid. Suitable body fluids include urine, serum, and synovial fluid. The preferred body fluid is urine.

Since the concentration of urinary peptides will decrease as the volume of urine increases, it is further preferred that when urine is the body fluid selected, the aliquot assayed be from a combined pool of urine collected over a fixed period of time, for example, 24 hours. In this way, the absolute rate of bone resorption is calculated for a 24 hour period. Alternatively, urinary peptides may be measured as a ratio relative to a marker

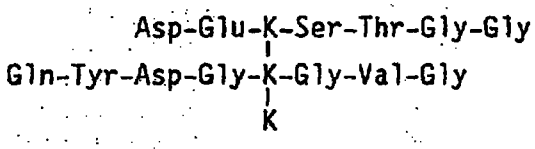
substance found in urine, such as creatinine. In this way the urinary index of bone resorption would remain independent of urine volume.

In one embodiment of the present invention, monoclonal or polyclonal antibodies are produced which are specific to the peptide fragments containing lysyl pyridinoline cross-links found in urine. Peptide fragments may be isolated from the urine of any patient; however, it is preferred that these peptides are isolated from patients with Paget's disease or hyperparathyroidism, due to the high concentration of peptide fragments found in these patients.

ISOLATION OF URINARY PEPTIDES

Urine from patients with active Paget's disease is dialyzed in reduced porosity dialysis tubing (>3,500 Spectropore™) at 4°C for 48h to remove bulk solutes. Under these conditions the peptides of interest are largely retained. The freeze-dried non-diffusate is then eluted (200 mg aliquots) from a column (90 cm x 2.5 cm) of Bio-Gel™ P2 (200-400 mesh) in 10% acetic acid at room temperature. A region of effluent that combines the cross-linked peptides is defined by measuring the fluorescence of collected fractions at 297 nm excitation/395 nm emission, and this pool is freeze-dried. Further resolution of this material is obtained on a column of Bio-Gel™ P-4 (200-400 mesh, 90 cm x 2.5 cm) eluted in 10% acetic acid. Two contiguous fraction pools are defined by monitoring the fluorescence of the eluant above. The earlier fraction is enriched in peptide fragments having two amino acid sequences that derive from the carboxyterminal telopeptide domain of the αI(I) chain of bone type I collagen linked to a third sequence derived from the triple-helical body of bone type I collagen. These three peptide sequences are cross-linked with 3-hydroxypyridinium. The overlapping later fraction is enriched in peptide fragments having an amino acid sequence that derives from the aminoterminal telopeptide domain of bone type I collagen linked through a 3-hydroxypyridinium cross-links. Individual peptides are then resolved from each of the two fractions obtained above by ion-exchange HPLC on a TSK DEAE-5-PW column (Bio Rad™ 7.5 cm x 7.5 mm) eluting with a gradient of NaCl (0-0.2M) in 0.02M Tris-HCl, pH 7.5 containing 10% (v/v) acetonitrile. The aminoterminal telopeptide-based and carboxyterminal telopeptide-based cross-linked peptides elute in a series of 3-4 peaks of fluorescence between 0.08M and 0.15M NaCl. The carboxyterminal telopeptide-based cross-linked peptides elute first as a series of fluorescent peaks, and the major and minor aminoterminal telopeptide-based cross-linked peptides elute toward the end of the gradient as characteristic peaks. Each of these is collected, freeze-dried and chromatographed on a C-18 reverse phase HPLC column (Vydac 218TP54, 25 cm x 4.6 mm) eluted with a gradient (0-10%) of acetonitrile: n-propanol (3:1 v/v) in 0.01M trifluoroacetic acid. About 100-500 µg of individual peptide fragments containing 3-hydroxypyridinium cross-links can be isolated by this procedure from a single 24h collection of Paget's urine. Amino acid compositions of the major isolated peptides confirmed purity and molecular sizes by the whole number stoichiometry of recovered amino acids. Aminoterminal sequence analysis by Edman degradation confirmed the basic core structures suspected from the sequences of the known cross-linking sites in type I collagen and from the matching amino acid compositions. The aminoterminal telopeptide sequence of the α2(I) chain was blocked from sequencing analysis due presumably to the known cyclization of the aminoterminal glutamine to pyrrolidone carboxylic acid. A typical elution profile of aminoterminal telopeptides obtained by the above procedure is shown in FIGURE 3a. The major peptide fragment obtained has an amino acid composition: $(Asx)_2(Glx)_2(Gly)_5$Val-Tyr-Ser-Thr, where Asx is the amino acid Asp or Asn and Glx is the amino acid Gln or Glu. The sequence of this peptide is represented by Formula III below. Normal urine contains smaller amounts of the peptide fragment represented by Formula III than the urine of Paget's disease patients.

FORMULA III



where

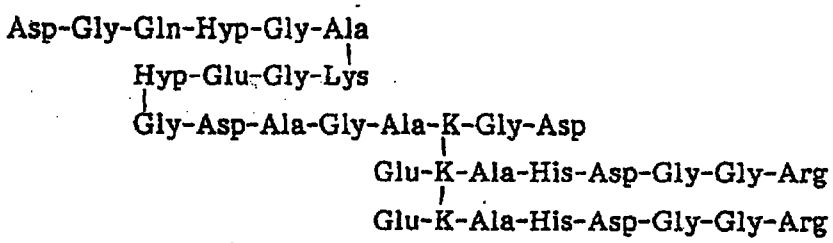


represents the HP or LP cross-linking amino acids, and

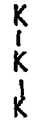
Gln represents glutamine or a wholly cyclized pyrrolidone carboxylic acid.

The carboxyterminal telopeptide-based cross-linked peptides resolved by reverse phase HPLC as described above are shown in FIGURE 3b. As can be seen from this figure, these peptides are further resolved into a series of carboxyterminal telopeptides each containing the 3-hydroxypyridinium cross-links. The major peptide, shown in FIGURE 3b, was analyzed as described above and was found to have the amino acid composition:
$(Asp)_5(Glu)_4(Gly)_{10}(His)_2(Arg)_2(Hyp)_2(Ala)_5$. The sequence of this peptide is represented by formula IV below. It is believed that the other carboxyterminal telopeptide-based cross-linked peptides appearing as minor peaks in FIGURE 3b represent additions and deletions of amino acids to the structure shown in Formula IV. Any of the peptides contained within these minor peaks are suitable for use as immunogens as described below.

FORMULA IV



where



represents the HP or LP cross-linking amino acids, and

Gln represents glutamine or a wholly cyclized pyrrolidone carboxylic acid.

Equivalents of the peptides represented by the above structures include those cases where some variation in the urinary peptide structure accrues. Examples of variation include amino acid additions to the N and C termini of Formulae III and IV as well as some terminal amino acid deletions. Smaller peptide fragments of the molecule represented by Formula IV derived from bone readsorption are especially evident in urine. These are found in the minor peaks of the carboxytelopeptide fraction seen in Figure 3b and can be identified by amino acid composition and sequence analysis. It is anticipated that antibodies produced to the haptens represented by Formulae III and IV will cross react with urinary peptides of slightly varied structure. In some situations it may be desirable to produce patient-specific antibodies to the urinary peptides derived from bone resorption. In these cases the same procedure described above is utilized to isolate urinary peptides whose structure may vary slightly from that represented by Formulae III and IV.

IMMUNOLOGICAL PROCEDURE FOR INDEXING BONE RESORPTION

Immunological binding partners capable of specifically binding to peptide fragments derived from bone collagen obtained from a physiological fluid can be prepared by methods well known in the art. The preferred method for isolating these peptide fragments is described above. By immunological binding partners as used

herein is meant antibodies and antibody fragments.

Both monoclonal and polyclonal antibodies specifically binding the peptides represented by Formulae III and IV and their equivalents are prepared by methods known in the art. For example, Laboratory Techniques in Biochemistry and Molecular Biology, Campbell, A. M. (1986) Vol. 13 Elsevier, herein incorporated by reference. It is possible to produce antibodies to the above peptides or their equivalents as isolated. However, because the molecular weights of these peptide fragments are less than 5,000, it is preferred that the hapten be conjugated to a carrier molecule. Suitable carrier molecules include, but are not limited to, bovine serum albumin, ovalbumin, thyroglobulin, and keyhole limpet hemocyanin (KLH). Preferred carriers are thyroglobulin and KLH.

It is well known in the art that the orientation of the hapten, as it is bound to the carrier protein, is of critical importance to the specificity of the anti-serum. Furthermore, not all hapten-protein conjugates are equally successful immunogens. The selection of a protocol for binding the particular hapten to the carrier protein therefore depends on the amino acid sequence of the urinary peptide fragments selected. For example, if the urinary peptide fragment represented by Formula III is selected, a preferred protocol would involve coupling this hapten to keyhole limpet hemocyanin (KLH), or other suitable carrier, with carbodiimide. This would ensure that most of the hapten would be conjugated through the Gly carboxyterminus, thereby presenting the preferred epitope, namely Tyr and 3-hydroxypyridinium cross-link, to the primed vertebrate antibody producing cells (e.g., B-lymphocytes).

Other urinary peptide fragments, depending on the source, may require different binding protocols. Accordingly, a number of binding agents may be suitably employed. These include, but are not limited to, carbodiimides, glutar-aldehyde, mixed anhydrides, as well as both homobifunctional and heterobifunctional reagents (see for example the Pierce 1986-87 catalog, Pierce Chemical Co., Rockford, IL). Preferred binding agents include carbodiimides and heterobifunctional reagents such as m-Maleimidobenzyl-N-hydroxysuccinimide ester (MBS).

Methods for binding the hapten to the carrier molecule are known in the art. See for example Laboratory Techniques in Biochemistry and Molecular Biology, Chard, T. (1987) Vol. 6, Partz Elsevier, N.Y., herein incorporated by reference.

Either monoclonal or polyclonal antibodies to the hapten-carrier molecule immunogen can be produced. However, it is preferred that monoclonal antibodies (MAb) be prepared. For this reason it is preferred that immunization be carried out in the mouse. Immunization protocols for the mouse usually include an adjuvant. Examples of suitable protocols are described by Chard, T. (1987) vida supra. Spleen cells from the immunized mouse are harvested and homogenized and thereafter fused with cancer cells in the presence of polyethylene glycol to produce a fused cell hybrid which produces monoclonal antibodies specific to peptide fragments derived from bone collagen. Examples of such peptide fragments are represented by Formulae III and IV above. Suitable cancer cells include myeloma, hepatoma, carcinoma, and sarcoma cells. Detailed descriptions of this procedure, including screening protocols, protocols for growing selected hybrid cells, and harvesting monoclonal antibodies produced by the selected hybrid cells are provided in Galfre, G. and Milstein, C. (1981) Meth. Enzymol. 73, 1. A preferred preliminary screening protocol involves the use of peptide fragments derived from bone collagen resorption and containing 3-hydroxypyridinium cross-links in a solid phase radioimmunoassay.

Immunological binding partners, especially monoclonal antibodies, produced by the above procedures, or equivalent procedures, are employed in various immunometric assays to quantitate the concentration of peptide fragments having 3-hydroxypyridinium cross-links derived from bone collagen resorption in body fluids. These immunometric assays comprise a monoclonal antibody or antibody fragment coupled to a detectable marker. Examples of suitable detectable markers include, but are not limited to, enzymes, coenzymes, enzyme inhibitors, chromophores, fluorophores, chemiluminescent materials, paramagnetic metals, spin labels, and radionuclides. Examples of standard immunometric methods suitable for indexing bone resorption include, but are not limited to, enzyme linked immunosorbent assay (ELISA) (Ingvall, E. (1981) Meth. Enzymol. 70), radioimmunoassay (RIA), and "sandwich" Immuno radiometric assay (IRMA). In its simplest form, these immunometric methods can be used to determine the absolute rate of bone resorption by simply contacting a body fluid with the immunological binding partner specific to a peptide fragment having 3-hydroxy-pyridinium cross-links derived from bone collagen resorption. It is preferred that the immunometric assays described above be conducted directly on untreated body fluids. Occasionally, however, contaminating substances may interfere with the assay, necessitating partial purification of the body fluid. Partial purification procedures include, but are not limited to, cartridge adsorption and elution, molecular sieve chromatography, dialysis, ion exchange, alumina chromatography, hydroxyapatite chromatography, and combinations thereof.

Test kits, suitable for use in accordance with the present invention, contain monoclonal antibodies prepared as described above that specifically bind to peptide fragments having 3-hydroxypyridinium cross-links derived from bone collagen resorption found in a body fluid. It is preferred that the monoclonal antibodies of this test

kit be coupled to a detectable marker of the type described above.

ELECTROCHEMICAL PROCEDURE FOR INDEXING BONE RESORPTION

An alternative procedure for indexing bone resorption consists of measuring a physical property of the peptide fragments having 3-hydroxypyridinium cross-links. One such physical property suitable for indexing bone resorption relies upon electrochemical detection. This method consists of injecting an aliquot of a body fluid, such as urine, into an electrochemical detector poised at a redox potential suitable for detection of peptides containing the 3-hydroxypyridinium ring. The 3-hydroxypyridinium ring, being a phenol, is subject to reversible oxidation, and therefore the electrochemical detector (e.g., Model 5100A Coulochem sold by esa 45 Wiggins Ave., Bedford, MA) is a highly desirable instrument suitable for quantitating the concentration of urinary peptides derived from bone adsorption. Two basic forms of electrochemical detector are currently commercially available: amperometric (e.g., BioAnalytical Systems) and coulometric (ESA, Inc., Bedford, MA 01730). Both are suitable for use in accordance with the present invention; however, the latter system is inherently more sensitive and therefore preferred since complete oxidation or reduction of the analyzed molecule in the column effluent is achieved. In addition, screening or guard electrodes can be placed "upstream" from the analytical electrode to selectively oxidize or reduce interfering substances thereby greatly improving selectivity. Essentially, the voltage of the analytical electrode is tuned to the redox potential of the sample molecule, and one or more pre-treatment cells are set to destroy interferents in the sample. In a preferred assay method, a standard current/voltage curve is established for standard peptides containing lysyl pyridinoline or hydroxylysyl pyridinoline in order to determine the proper voltage to set for optimal sensitivity. This voltage is then modified, depending upon the body fluid, to minimize interference from contaminants and optimize sensitivity. Electrochemical detectors, and the optimum conditions for their use are known to those skilled in the art. Complex mixtures of body fluids can often be directly analyzed with the electrochemical detector without interference. Accordingly, for most patients no pretreatment of the body fluid is necessary. In some cases, however, interfering compounds may reduce the reliability of the measurements. In such cases, pretreatment of the body fluid (e.g., urine) may be necessary.

Accordingly, in an alternative embodiment of the invention, a body fluid is first purified prior to electrochemically titrating the purified peptide fragments. The purification step may be conducted in a variety of ways, including but not limited to, dialysis, ion exchange chromatography, alumina chromatography, hydroxyapatite chromatography, molecular sieve chromatography, or combinations thereof. In a preferred purification protocol, a measured aliquot (25 ml) of a 24 hour urine sample is dialyzed in reduced porosity dialysis tubing to remove the bulk of contaminating fluorescent solutes. The non-diffusate is then lyophilized, redissolved in 1% heptafluorobutyric acid (HFBA), an ion pairing solution, and the peptides adsorbed on a Waters Sep-Pak C-18 cartridge. This cartridge is then washed with 5 ml of 1% HFBA, and then eluted with 3 ml of 50% methanol in 1% HFBA.

Another preferred method of purification consists of adsorbing a measured aliquot of urine onto an ion-exchange adsorption filter and eluting the adsorption filter with a buffered eluting solution. The eluate fractions containing peptide fragments having 3-hydroxypyridinium cross-links are then collected to be assayed.

Still another preferred method of purification employs molecular sieve chromatography. For example, an aliquot of urine is applied to a Bio-Gel™ P2 or Sephadex G-20 column, and the fraction eluting in the 1000-5000 Dalton range is collected. It will be obvious to those skilled in the art that a combination of the above methods may be used to purify or partially purify urine or other body fluids in order to isolate the peptide fragments having 3-hydroxypyridinium cross-links. The purified or partially purified peptide fragments obtained by the above procedures may be subjected to additional purification procedures, further processed, or assayed directly in the partially purified state. Additional purification procedures include resolving partially purified peptide fragments employing high performance liquid chromatography (HPLC) or microbore HPLC when increased sensitivity is desired. These peptides may then be quantitated by electrochemical titration. A preferred electrochemical titration protocol consists of tuning the redox potential of the detecting cell of the electrochemical detector (Coulochem Model 5100A) for maximum signal with pure HP. The detector is then used to monitor the effluant from a C-18 HPLC column used to resolve the partially purified urinary peptides.

FLUOROMETRIC PROCEDURE FOR INDEXING BONE RESORPTION

An alternative preferred method for quantitating the concentration of peptide fragments having 3-hydroxypyridinium cross-links is to measure the characteristic natural fluorescence of these peptide fragments. For those body fluids containing few naturally occurring fluorescent materials other than the 3-hydroxypyridinium cross-links, fluorometric assay may be conducted directly without further purification of the body fluid. In this

case, peptide fragments are resolved by HPLC and the natural fluorescence of the HP and LP amino acid residues is measured at 395 nm upon excitation at 297 nm, essentially as described by Eyre, D.R., et al., Analyl. Biochem. 137, 380 (1984), herein incorporated by reference.

It is preferred, in accordance with the present invention, that the fluorometric assay be conducted on urine. Urine, however, usually contains substantial amounts of naturally occurring fluorescent contaminants that must be removed prior to conducting the fluorometric assay. Accordingly, urine samples are first partially purified as described above for electrochemical detection. This partially purified urine sample can then be fluorometrically assayed as described above.

Optionally, the body fluid (preferably urine) is passed directly through a C-18 reverse phase affinity cartridge after adding acetonitrile/methanol 5 to 10% V/V. The non-retentate is adjusted to 0.05-0.10M with a cationic ion-pairing agent such as tetrabutyl ammonium hydroxide and passed through a second C-18 reverse phase cartridge. The washed retentate, containing fluorescent peptides, from this second cartridge is eluted with acetonitrile:water (or methanol:water), and dried, and the fluorescent peptides are analyzed by reverse phase HPLC or microbore HPLC using an anionic ion-pairing agent such as 0.01M trifluoroacetic acid in the eluant. Alternatively or included in this peptide clean-up procedure, a molecular sieve step employing a Bio-Gel P2 (Bio-Rad labs) or equivalent gel filtration medium, can be used.

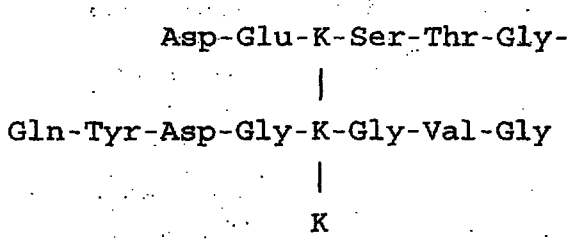
FIGURE 4A displays the elution profile resolved by reverse phase HPLC of natural fluorescence for a hydrolysate of peptide fragments from normal human urine. Measurement of the integrated area within the envelope of a given component is used to determine the concentration of that component within the sample. The ratio of HP:LP found in normal human urine and urine from patients having Paget's disease, FIGURE 4B, are both approximately 4.5:1. This is slightly higher than the 4:1 ratio found in bone itself (Eyre et al., 1984). The higher ratio found in urine indicates that a portion of the HP fraction in urine may come from sources other than bone such as the diet, or other sources of collagen degradation; i.e., cartilage catabolism. It is for this reason that it is preferred that peptide fragments having LP crosslinks, which derives only from bone, be used to provide an absolute index of bone resorption. However, in the absence of excessive cartilage degradation such as in rheumatoid arthritis or in cases where bone is rapidly being absorbed, peptide fragments having HP crosslinks, or a combination of peptide fragments having HP crosslinks, plus peptide fragments having LP crosslinks, may be used as an index of bone resorption.

While the invention has been described in conjunction with preferred embodiments, one of ordinary skill after reading the foregoing specification will be able to effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein. Hence, the invention can be practiced in ways other than those specifically described herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the appended claims and equivalents thereof.

Claims

1. A method of determining the rate of bone resorption, the method comprising quantitating the concentration of a peptide fragment, in a body fluid, which is derived from bone collagen resorption and has a 3-hydroxypyridinium cross-link that is lysyl pyridinoline and/or hydroxylysyl pyridinoline.

2. A method according to claim 1 wherein the peptide fragment is derived from the aminoterminal telopeptide domain of bone type I collagen linked through a 3-hydroxypyridinium cross-link which comprises the amino acid sequence:



where

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case, peptide fragments are resolved by HPLC and the natural fluorescence of the HP and LP amino acid residues is measured at 395 nm upon excitation at 297 nm, essentially as described by Eyre, D.R., et al., Analyl. Biochem. 137, 380 (1984), herein incorporated by reference.

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Optionally, the body fluid (preferably urine) is passed directly through a C-18 reverse phase affinity cartridge after adding acetonitrile/methanol 5 to 10% V/V. The non-retentate is adjusted to 0.05-0.10M with a cationic ion-pairing agent such as tetrabutyl ammonium hydroxide and passed through a second C-18 reverse phase cartridge. The washed retentate, containing fluorescent peptides, from this second cartridge is eluted with acetonitrile:water (or methanol:water), and dried, and the fluorescent peptides are analyzed by reverse phase HPLC or microbore HPLC using an anionic ion-pairing agent such as 0.01M trifluoroacetic acid in the eluant. Alternatively or included in this peptide clean-up procedure, a molecular sieve step employing a Bio-Gel P2 (Bio-Rad labs) or equivalent gel filtration medium, can be used.

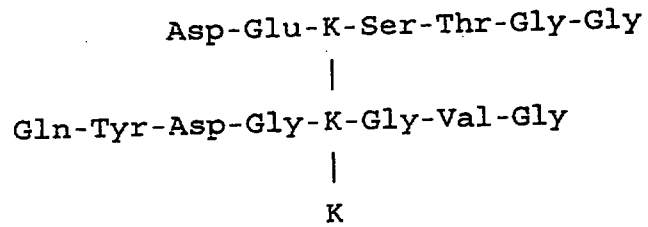
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While the invention has been described in conjunction with preferred embodiments, one of ordinary skill after reading the foregoing specification will be able to effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein. Hence, the invention can be practiced in ways other than those specifically described herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the appended claims and equivalents thereof.

Claims

1. A method of determining the rate of bone resorption, the method comprising quantitating the concentration of a peptide fragment, in a body fluid, which is derived from bone collagen resorption and has a 3-hydroxypyridinium cross-link that is lysyl pyridinoline and/or hydroxylysyl pyridinoline.

2. A method according to claim 1 wherein the peptide fragment is derived from the aminoterminal telopeptide domain of bone type I collagen linked through a 3-hydroxypyridinium cross-link which comprises the amino acid sequence:



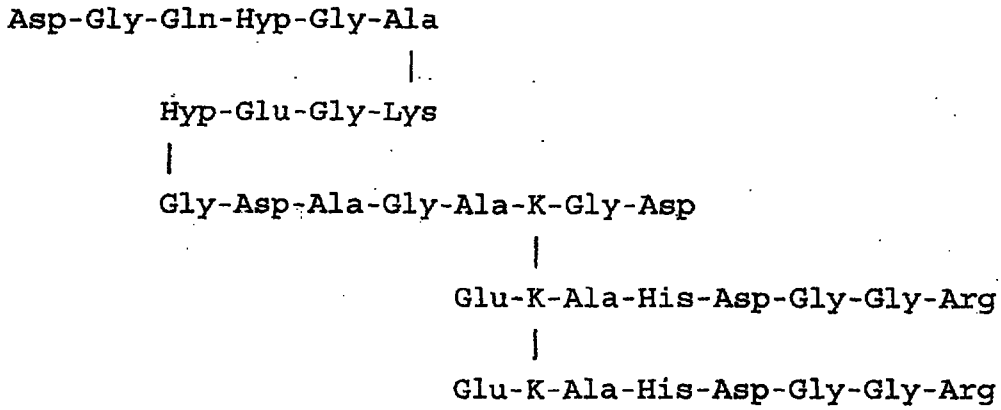
where

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represents hydroxylysyl pyridinoline or lysyl pyridinoline, and
Gln is glutamine or wholly cyclized pyrrolidone carboxylic acid.

3. A method according to claim 1 wherein the peptide fragment is derived from the carboxy terminal telopeptide domain of bone type I collagen linked through a 3-hydroxypyridinium cross-link which comprises the amino acid sequence:



where



represents hydroxylysyl pyridinoline or lysyl pyridinoline.

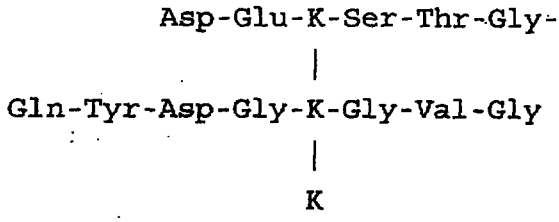
4. A method as claimed in any preceding claim wherein the body fluid is urine, serum or synovial fluid.

5. A method as claimed in any preceding claim wherein quantitating comprises contacting the body fluid with an immunological binding partner specific to the peptide fragment having a 3-hydroxypyridinium cross-link derived from bone collagen resorption.

6. A method as claimed in claim 5 further comprising, prior to contacting the body fluid, purifying the body fluid.

7. A method as claimed in claim 6 wherein the purifying step is cartridge adsorption and elution, molecular sieve chromatography, dialysis, ion exchange, alumina chromatography and/or hydroxyapatite chromatography.

8. A peptide fragment, derived from the aminoterminal telopeptide domain of bone type I collagen linked through a 3-hydroxypyridinium cross-link, comprising the amino acid sequence:

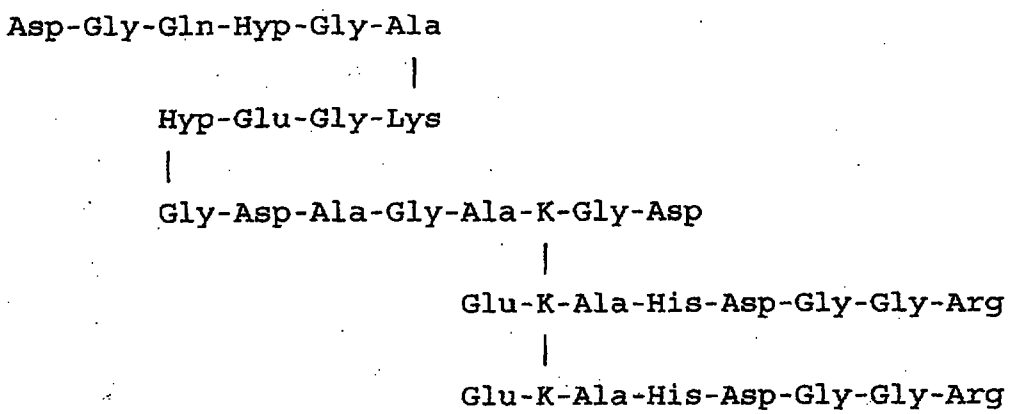


where



represents hydroxylysyl pyridinoline or lysyl pyridinoline, and
Gln is glutamine or wholly cyclized pyrrolidone carboxylic acid.

9. A peptide fragment derived from the carboxy terminal telopeptide domain of bone type I collagen linked through a 3-hydroxypyridinium cross-link, comprising the amino acid sequence:



where



represents hydroxylysyl pyridinoline or lysyl pyridinoline.

10. A hybridoma capable of producing monoclonal antibodies specific for the peptide fragment of claim 8 or 9.

11. An immunological binding partner specific for a peptide fragment as defined in claim 8 or 9.

12. An immunological binding partner according to claim 11 which is a monoclonal antibody.

13. A immunological binding partner as claimed in claim 11 or 12 coupled to a detectable marker.

14. A test kit for quantitating the amount of a peptide fragment in a body fluid derived from bone collagen resorption having a 3-hydroxypyridinium cross-link the kit comprising an immunological binding partner as defined in any of claims 11 to 13.

15. The use of a peptide fragment as claimed in claim 8 or 9 in the preparation of an immunogenic agent.

16. The use of peptide fragment as claimed in claim 8 or 9 for use as a standard in quantitating the amount of that peptide fragment in a body fluid derived from bone collagen resorption.

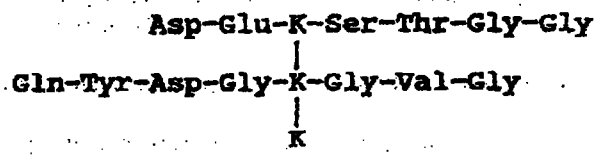
17. An immunological binding partner as claimed in any of claims 11 to 13 for use as a diagnostic agent.

18. An immunogenic composition comprising a peptide fragment as defined in claim 8 or 9.

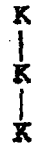
Patentansprüche

1. Verfahren zur Bestimmung der Geschwindigkeit von Knochenresorption, welches Verfahren die quantitative Bestimmung der Konzentration eines Peptidfragments in einer Körperflüssigkeit umfaßt, das aus der Knochenkollagenresorption stammt und eine 3-Hydroxypyridinium-Vernetzungsstelle aufweist, die Lysyl-pyridinolin und/oder Hydroxylysyl-pyridinolin ist.

2. Verfahren nach Anspruch 1, worin das Peptidfragment von der aminoterminalen Telopeptid-Domaine von Knochen-Typ-I-Kollagen herrührt, welche durch eine 3-Hydroxypyridinium-Vernetzungsstelle vernetzt ist und die Aminosäuresequenz:

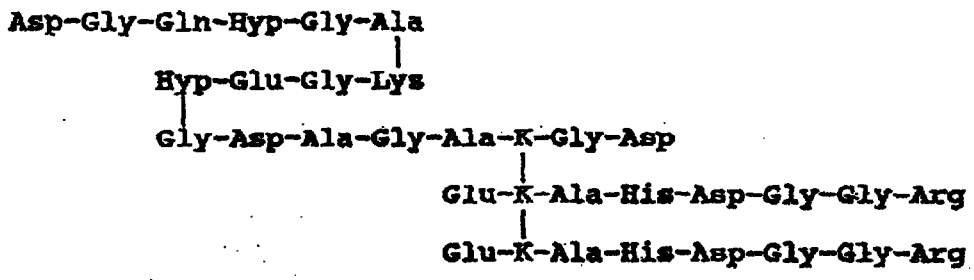


umfaßt, in der



Hydroxylysyl-pyridinolin oder Lysyl-pyridinolin repräsentiert und Gln Glutamin oder gänzlich cyclisierte Pyrrolidon-carbonsäure ist.

3. Verfahren nach Anspruch 1, worin das Peptidfragment von der carboxyterminalen Telopeptid-Domaine von Knochen-Typ-I-Kollagen herrührt, welche durch eine 3-Hydroxypyridinium-Vernetzungsstelle vernetzt ist und die Aminosäuresequenz:



umfaßt, in der



Hydroxylysyl-pyridinolin oder Lysyl-pyridinolin repräsentiert.

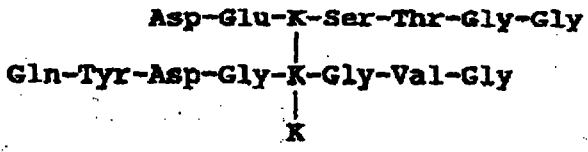
4. Verfahren nach irgendeinem vorhergehenden Anspruch, worin die Körperflüssigkeit Urin, Serum oder Gelenkflüssigkeit ist.

5. Verfahren nach irgendeinem vorhergehenden Anspruch, worin die quantitative Bestimmung umfaßt, daß die Körperflüssigkeit mit einem immunologischen Bindungspartner in Kontakt gebracht wird, welcher spezifisch ist für das aus der Knochenkollagenresorption stammende Peptidfragment mit einer 3-Hydroxypyridinium-Vernetzungsstelle.

6. Verfahren nach Anspruch 5, das außerdem umfaßt, daß die Körperflüssigkeit vor dem Inkontaktbringen gereinigt wird.

7. Verfahren nach Anspruch 6, worin der Reinigungsschritt Patronenadsorption und elution, Molekularsieb-Chromatographie, Dialyse, Ionenaustausch, Aluminiumoxid- und/oder Hydroxylapatitchromatographie ist.

8. Peptidfragment, das von der aminoterminalen Telopeptid-Domaine von Knochen-Typ-I-Kollagen herrührt, welche durch eine 3-Hydroxypyridinium-Vernetzungsstelle vernetzt ist und die Aminosäuresequenz:

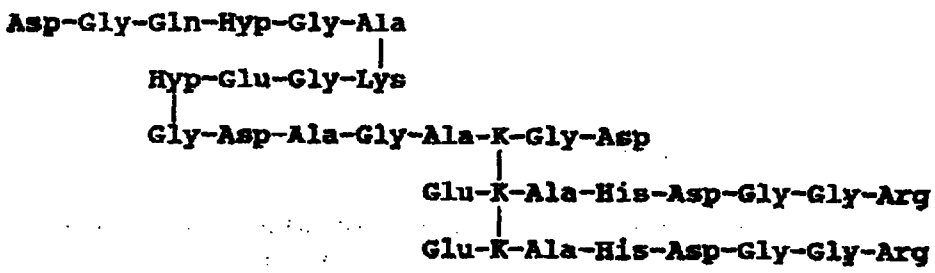


umfaßt, in der

Hydroxylysyl-pyridinolin oder Lysyl-pyridinolin repräsentiert und Gln Glutamin oder gänzlich cyclisierte Pyrrolidon-carbonsäure ist.

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9. Peptidfragment, das von der carboxyterminalen Telopeptid-Domaine von Knochen-Typ-I-Kollagen herrührt, welche durch eine 3-Hydroxypyridinium-Vernetzungsstelle vernetzt ist und die Aminosäuresequenz:



umfaßt, in der



Hydroxylysyl-pyridinolin oder Lysyl-pyridinolin repräsentiert.

10. Hybridom, das dazu fähig ist, monoklonale Antikörper zu erzeugen, welche spezifisch für das Peptidfragment von Anspruch 8 oder 9 sind.

11. Immunologischer Bindungspartner, der spezifisch für ein Peptidfragment ist, wie es in Anspruch 8 oder 9 definiert ist.

12. Immunologischer Bindungspartner nach Anspruch 11, der ein monoklonaler Antikörper ist.

13. Immunologischer Bindungspartner nach Anspruch 11 oder 12, der an einen auffindbaren Marker gekoppelt ist.

14. Test-Ausrüstung für die quantitative Bestimmung der Menge eines Peptidfragments in einer Körperflüssigkeit, das aus der Knochenkollagenresorption stammt und eine 3-Hydroxypyridinium-Vernetzungsstelle aufweist, wobei die Ausrüstung einen immunologischen Bindungspartner umfaßt, wie er in einem der Ansprüche 11 bis 13 definiert ist.

15. Verwendung eines Peptidfragments nach Anspruch 8 oder 9 bei der Herstellung eines immunogenen Agens.

16. Verwendung des Peptidfragments nach Anspruch 8 oder 9 als ein Standard bei der quantitativen Bestimmung der Menge jenes Peptidfragments in einer Körperflüssigkeit, welches aus der Knochenkollagenresorption stammt.

17. Immunologischer Bindungspartner nach irgendeinem der Ansprüche 11 bis 13 für die Verwendung als ein diagnostisches Mittel.

18. Immunogene Zusammensetzung, umfassend ein Peptidfragment, wie es in Anspruch 8 oder 9 definiert ist.

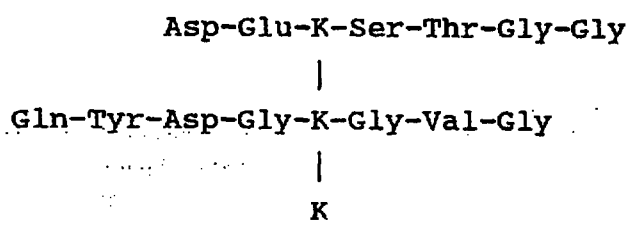
Revendications

1. Procédé de détermination de la vitesse de résorption osseuse, le procédé comprenant la quantification de la concentration d'un fragment peptidique dans un fluide du corps, qui dérive de la résorption osseuse

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du collagène et qui a une liaison croisée 3-hydroxypyridinium, qui est la lysylpyridinoline et/ou l'hydroxylysylpyridinoline.

2. Procédé suivant la revendication 1, dans lequel le fragment peptidique dérive de la région amino-terminale télopeptidique du collagène osseux de type I, lié par une liaison croisée 3-hydroxypyridinium, qui comprend la séquence en acides aminés :

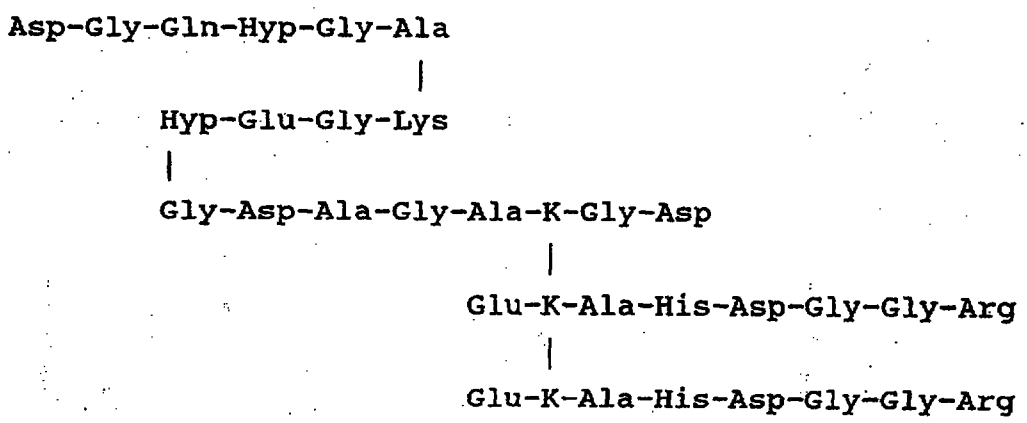


où



représente hydroxylysylpyridinoline ou lysylpyridinoline, et
Gln est glutamine ou acide pyrrolidonecarboxylique complètement cyclisé.

3. Procédé suivant la revendication 1, dans lequel le fragment peptidique dérive de la région carboxy-terminale télopeptidique du collagène osseux de type I lié par une liaison croisée 3-hydroxypyridinium, qui comprend la séquence en acides aminés :



où

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K
|
K
|
K

représente hydroxylysylpyridinoline ou lysylpyridinoline.

4. Procédé suivant l'une quelconque des revendications précédentes, dans lequel le fluide du corps est l'urine, du sérum ou du fluide synovial.

5. Procédé suivant l'une quelconque des revendications précédentes, dans lequel la quantification comprend la mise en contact du fluide du corps avec un partenaire de liaison immunologique spécifique du fragment peptidique ayant une liaison croisée 3-hydroxypyridinium, dérivé de la résorption osseuse du collagène.

6. Procédé suivant la revendication 5, comprenant de plus, avant l'étape de mise en contact du fluide du corps, la purification du fluide du corps.

7. Procédé suivant la revendication 6, dans lequel l'étape de purification consiste en une cartouche d'adsorption et d'élution, une chromatographie sur tamis moléculaire, une dialyse, un échange d'ions, une chromatographie sur alumine et une chromatographie sur hydroxyapatite.

8. Fragment peptidique dérivé de la région amino-terminale télopeptidique du collagène osseux de type I, lié par une liaison croisée 3-hydroxypyridinium, comprenant la séquence en acides aminés :

Asp-Glu-K-Ser-Thr-Gly-Gly
|
Gln-Tyr-Asp-Gly-K-Gly-Val-Gly
|
K

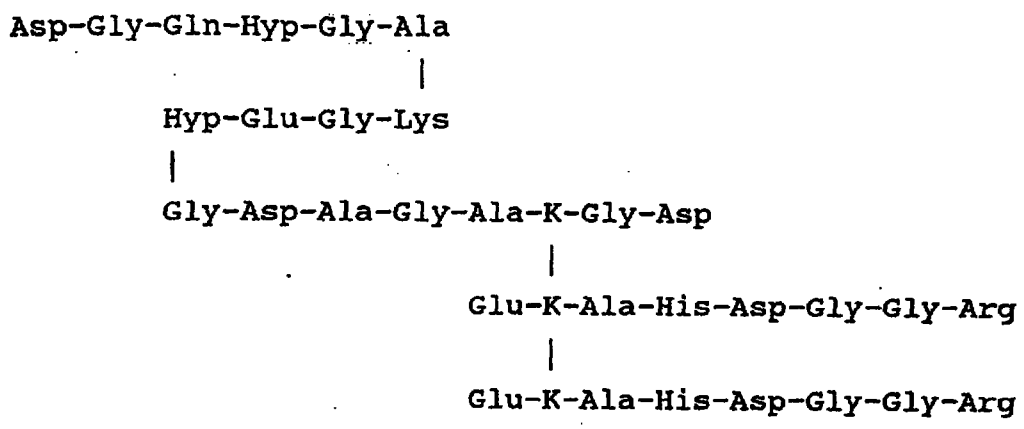
où

K
|
K
|
K

représente hydroxylysylpyridinoline ou lysylpyridinoline, et
Gln est glutamine ou acide pyrrolidonecarboxylique complètement cyclisé.

9. Fragment peptidique dérivé de la région carboxy-terminale télopeptidique du collagène osseux de type I, lié par une liaison croisée 3-hydroxypyridinium, comprenant la séquence en acides aminés :





où



représente hydroxylysylpyridinoline ou lysylpyridinoline.

10. Hybridome capable de produire des anticorps monoclonaux spécifiques du fragment peptidique de la revendication 8 ou 9.

11. Partenaire de liaison immunologique spécifique d'un fragment peptidique tel que défini à la revendication 8 ou 9.

12. Partenaire de liaison immunologique suivant la revendication 11, qui est un anticorps monoclonal.

13. Partenaire de liaison immunologique suivant la revendication 11 ou 12, couplé à un marqueur détectable.

14. Equipement d'épreuve pour déterminer la quantité d'un fragment peptidique dans un fluide du corps, dérivé de la résorption osseuse du collagène et ayant une liaison croisée 3-hydroxypyridinium, l'équipement comprenant un partenaire de liaison immunologique tel que défini à l'une quelconque des revendications 11 à 13.

15. Utilisation d'un fragment peptidique suivant la revendication 8 ou 9, dans la préparation d'un agent immunogène.

16. Utilisation du fragment peptidique suivant la revendication 8 ou 9, comme standard dans la quantification de la quantité de ce fragment peptidique dans un fluide du corps dérivé de la résorption osseuse du collagène.

17. Partenaire de liaison immunologique suivant l'une quelconque des revendications 11 à 13, à utiliser comme agent diagnostique.

18. Composition immunogénique comprenant un fragment peptidique tel que défini à la revendication 8 ou 9.