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(54) NEW HYPOCALCAEMIC PEPTIDES AND PROCESS FOR THEIR MANUFACTURE

- (71) We, CIBA-GEIGY AG, formerly known as Ciba Limited, a body corporate organised according to the laws of Switzerland, of Basle, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—
- This invention relates to hypocalcaemic peptides and to a process for their manufacture.
- It is known that hypocalcaemic hormones occur in mammals and in humans. The structure of porcine thyrocalcitonin has already been explored; it is a dotriacontapeptide amide containing a cystine disulfide ring at the N-terminus. In view of the great importance of calcitonin in calcium metabolism, in the formation of bones and in relevant therapy it is important to have human thyrocalcitonin available in pure form.
- The present invention provides a process for the manufacture of human calcitonin in pure form from C-cell rich medulla carcinoma of the thyroid gland or from C-cell metastasis material wherein medullar carcinoma of the thyroid gland or C-cell metastasis material, which has been de-fatted, for example with acetone or ether, and which may have been first purified with alcohol or with aqueous trichloroacetic acid, is extracted one or more times with a solvent system containing water and an alkanol having at most 5 carbon atoms, at a pH of from about 1 to 6, and the extracted product subjected to gel chromatography using aqueous formic acid as eluant. The calcitonin may be separated into its constituents by counter current distribution, for example by Craig distribution using a solvent system advantageously containing n-butanol and acetic acid.
- The present invention also provides human calcitonin in the form of its components HT—C₁, HT—C₂, HT—C₁₁ and/or HT—C₂₁ or an acid addition salt or complex thereof.
- The solvent system used for the extraction contains as alkanol, for example methanol, ethanol, propanol, n-butanol or secondary butanol. The water content of the system may vary, for example, from 4 to 50%, preferably from 5 to 30%. The above-mentioned acid pH value is established, for example, with an inorganic acid such as sulfuric or hydrochloric acid, or with an organic acid such as formic or acetic acid, or with an acid buffer such as acetate or citrate buffer. If desired, the solvent system may contain further organic solvents such as acetone, or organic bases such as pyridine or morpholine, and/or inorganic salts such as sodium chloride. Particularly useful are systems containing n-butanol and acetic acid, especially n-butanol + glacial acetic acid + water (75:7.5:21).
- The extraction may be performed in several stages with the same or with different solvent systems.
- The crude product is obtained from the extract, for example by evaporation, lyophilization or precipitation, for instance with acetone or trichloroacetic acid.
- The chromatographic purification is advantageously carried out on Sephadex G-50 (Sephadex is a Registered Trade Mark), on Bio Gel P6 or on an equivalent material. Sephadex G-50 is a cross-linked polydextran gel with a fractionation range of molecular

[Price 25p]

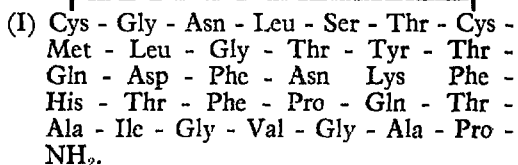
weights of from 500 to 10,000. Bio gel P-6 is a polyacrylamide gel with a fractionation range of molecular weights of from 1000 to 5000. The elution is performed, for example, with 0.1 N-formic acid.

As mentioned above, Craig distribution is advantageously carried out with systems containing n-butanol and acetic acid, for example n-butanol+glacial acetic acid+water (4:1:5).

The main component of human calcitonin (designated herein as HT-C₂) is a dotriacontapeptide amide with the following aminoacid sequence: Lys (1), His (1), Asp (3), Thr (5), Ser (1), Glu (2), Pro (2), Gly (4), Ala (2), 1/2 Cys (2), Val (1), Met (1), Ile (1), Leu (2), Tyr (1), Phe (3), 4 of the 5 carboxylic side-chains being in amide form. In addition to it, further active components which are referred to below by HT-C₁, HT-C₁₁ and HT-C₂₁ have been isolated in pure form. The aminoacid analysis of HT-C₁ is identical with that of HT-C₂. Thus, the main component of human calcitonin differs considerably in its aminoacid sequence from that of porcine thyrocalcitonin. When HT-C₁ and HT-C₂ are enzymatically split with trypsin they form two fragments Tr_I and Tr_{II} having the aminoacid sequence indicated in Table 1 in Example 1.

The components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁ are unitary in thin layer chromatography and electrophoresis.

It was found that HT-C₂ corresponds to the following structural formula:



HT-C₁ is the dimer of this compound. From the fact that on dansylation of HT-C₁ and acid hydrolysis no bis-dansyl-cystine is formed, one can conclude that HT-C₁ is an anti-parallel dimer.

Under basic conditions, for example with dilute ammonia, or by reduction, for instance with dimercaptoethanol or dithiothreitol in aqueous pyridine at room temperature, HT-C₁ is converted into HT-C₂.

The components HT-C₁ and HT-C₂ can be converted into their sulfoxides by known oxidation methods, for example with aqueous hydrogen peroxide. The characterization of the sulfoxides is shown in the Examples.

Human calcitonin (components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁, individually or in any desired mixture) reveals in the rat an activity of about 100 to 200 MRC units per mg (peptide) in the test described by Kumar et al, J. Endocrinology 33, page

470 [1965]. It lowers the plasma calcium level and plasma phosphate level of the blood of mammals, as can be shown by tests on rats weighing 50 to 150 g. In patients with increased bone turn over it lowers the calcium level of the blood on intravenous, intramuscular or subcutaneous injection of 0.01 to 5 mg, for instance in 0.1 molar acetate buffer of pH 4.6. It may therefore be used for the treatment of hypocalcaemia and of bone diseases such as Paget's disease or osteoporosis.

HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁ are bases capable of forming acid addition salts with inorganic or organic acid. The free bases can be liberated from the acid addition salts in known manner. For pharmaceutical purposes the base may be converted into physiologically tolerable acid addition salts, for example into salts with inorganic acids such as hydrohalic acids, for example hydrochloric or hydrobromic acid, nitric, thiocyanic, sulfuric or phosphoric acid, or with organic acids such as acetic, propionic, stearic, glycolic, lactic, pyruvic, oxalic, citric, benzoic, cinnamic, salicylic, 2-phenoxy- or 2-acetoxybenzoic acid, mandelic, methanesulfonic, ethanesulfonic, hydroxyethanesulfonic, benzene- or toluenesulfonic, trichloro- or trifluoroacetic acid.

Human calcitonin can be converted in known manner into pharmaceutically useful complexes having a prolonged activity, for example complexes with substances that are known to prolong the activity of peptides, such as nonantigenic gelatin, especially oxypolygelatin, polyvinyl-pyrrolidone, polyphosphatetinephosphate, phytic acid, protamine, polyglutamic acid or sparingly soluble metal compounds, for example phosphates, pyrophosphates or hydroxides of aluminum, magnesium, cobalt or especially zinc.

The salts and complexes are suitable for the same use as the free peptide.

The present invention also provides a pharmaceutical preparation containing one or more of the components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁ of human calcitonin, or a physiologically tolerable acid addition salt or complex thereof in admixture or conjunction with a pharmaceutically acceptable carrier suitable for use in enteral or parenteral administration. Suitable excipients are, for example, water, physiological saline solutions, gelatin, lactose, glucose, starches, vegetable oils, benzyl alcohols, polyalkyleneglycols or other known pharmaceutical carriers. The preparations may be sterilized and/or contain assistants such as preservatives, stabilizers, wetting agents or emulsifiers. They may also contain further therapeutically valuable additives.

The following Examples illustrate the invention.

In thin-layer chromatography the following systems are used:

System

- 5 45 sec-butanol+3% aqueous ammonia (7:3)
52 n-butanol+glacial acetic acid+water (75:7.5:21)
79 n-butanol+pyridine+water (1:1:1)
10 101A n-butanol+pyridine+glacial acetic acid+water (42:24:4:30)
104 chloroform+methanol+17% aqueous ammonia (41:41:18).

Unless otherwise indicated, thin-layer chromatography is carried out on Alox D-O (Messrs. Camag). All values (R_s) are referred to porcine α -thyrocalcitonin sulfoxide=1.

Electrophoresis is performed on Avicel (Registered Trade Mark) cellulose or on Cello-gel (cellulose acetate films) at pH=1.9 [using as buffer, glacial acetic acid+formic acid+water (95:26.5:980)] at a potential of 140 Volt for 2 hours. The distance travelled (d) during electrophoresis is referred to porcine α -thyrocalcitonin sulfoxide=1.

EXAMPLE 1

10 Grams of medulla carcinoma are immediately deep-frozen and lyophilized. The fatty constituents are removed by extraction with ether and the dry tissue (2g) is thoroughly extracted 3 times with 10 times the volume of a mixture of n-butanol+glacial acetic acid+water (75:7.5:21) at 20°C. The extracts are combined, *in vacuo* freed from butanol and lyophilized, to yield 450 mg of a dry residue having an activity of 12 MRC units per mg. 300 mg of this product in 0.1N-formic acid are purified by gel filtration through a column (450 ml) of Bio Gel P6 or Sephadex G-50 in 0.1N-formic acid. The active fractions (0.5 to 0.7 column volumes) are combined and lyophilized, to yield 18 mg of a product of 50 MRC units per mg. This material is then subjected to a multiplicative distribution in the system glacial acetic acid+water+butanol (1:5:4) over 140 stages, choosing a phase volume of 3 ml at a phase ratio of 1. The active fractions identified by testing are combined in groups of 10 elements each, and are freed from the solvent and lyophilized. For the fractions with maximum activity, $K=0.6$. The peptide HT-C₂ obtained therefrom is pure according to thin-layer chromatography and electrophoresis. In the rat it displays an activity of about 100—200 MRC units per mg of peptide. The distance travelled (d) in the system 52 on Alox DO is 1.16; in the system 79 on Alox DO it is 1.79; in the system 104 on Alox DO it is 1.28; in the system 45 on Alox DO it is 1.37. In glacial acetic acid+formic acid+water (95:26.5:980) at pH 1.9 the product travels (at a potential 140

volts for 2 hours) on Avicel cellulose towards the cathode by a distance $d=0.77$.

The product gives positive color reactions with ninhydrin, chlorotolidine, potassium hexacyanoferrate (II)-iron (III) chloride and diazotized sulfanilic acid amide. There is no reaction with 4-dimethylaminobenzaldehyde-hydrochloric acid or Sakaguchi reagent. There is no ultraviolet fluorescence and tryptophan is absent. The aminoacid analysis after acid hydrolysis gives the following result: Lysine (1), histidine (1), aspartic acid (3), threonine (5), serine (1), glutamic acid (2), proline (2), glycine (4), alanine (2), 1/2 cystine (2), valine (1), methionine (1), isoleucine (1), leucine (2), tyrosine (1), phenylalanine (3), NH₃. The sulf-oxide of HT-C₂ reveals the following values: R_s 52=1.07, R_s 45=1.20, R_s 79=1.66.

For further characterization the enzymatic splitting of the peptide with trypsin is used. There are obtained two fragments, namely Tr_I containing the aminoacids 1—18, and Tr_{II} with the aminoacids 19—32. The amino-acid sequence of the two fragments is shown in Table 1. Tr_{II} is in thin-layer chromatography on Alox DO distinctly more lipophilic than Tr_I and travels in the electrophoresis faster towards the cathode than does Tr_I.

Fragment Tr_I: R_s 52=0.53; R_s 79=0.54; R_s 104=0.51. Electrophoresis on Avicel cellulose: $d=0.81$.

Fragment Tr_{II}: R_s 52=1.38; R_s 79=1.45; R_s 104=1.43. Electrophoresis on Avicel cellulose: $d=0.94$.

TABLE 1

	Tr _I (3)	Tr _{II} (2)	
NH ₃	1		100
Lys			
His		1	
Asp	3		
Thr	3	2	105
Ser	1		
Glu	1	1	
Pro		2	
Gly	2	2	
Ala		2	110
1/2 Cys	2		
Val		1	
Met	1		
Ile		1	
Leu	2		115
Tyr	1		
Phe	1	2	
	18	14	

EXAMPLE 2

110 Grams of deep-frozen C-cell metastasis material taken from the chest are defatted with ether and then extracted as described in Example 1 with n-butanol+glacial

acetic acid+water (75:7.5:21), to furnish 6 g of dry extract as a dark-brown powder, which is repeatedly agitated with 0.1N-formic acid (total 150 ml), during which 3.6 g of the dry residue pass into solution. The solution is subjected in two portions on a Bio Gel P6 column (5 cm; 85 cm; volume about 1.6 liter) to gel chromatography with 0.1N-formic acid and collected in fractions of 20 ml each. There are two peaks with active material present, namely in fractions 13—21 with the peak maximum at 0.4 column volume and the fractions 25—34 with the peak maximum at 0.55 column volume. The fractions 25—34 are combined and lyophilized to furnish 105 mg of crude product which is identical with the product obtained by gel filtration as described in Example 1 and, after multiplicative distribution according to Example 1, furnishes pure HT—C. The combined fractions 13—21 are lyophilized and furnish 202 mg of crude product which is subjected to a multiplicative distribution in the system n-butanol+glacial acetic acid+water (4:1:5) over 400 stages in elements having a phase volume of 3 ml each. The activity maximum is at K=0.19. The resulting pure product (HT—C₁) gives in the rat test according to Kumar et al. (see above) about as good results as the product obtained in Example 1 (HT—C₂). It can be converted, for example with 5% hydrogen peroxide, into the equally active sulfoxide. In thin-layer chromatography on Alox DO Rs 52=1.04; Rs 79=1.42; Rs 45=1.20. The sulfoxide reveals the following values: Rs 52=0.88; Rs 79=1.29; Rs 45=1.09. In electrophoresis on Avicel cellulose d=0.90 for HT—C₁ and its sulfoxide. On Cellocl d=1.15 for HT—C₁ and d=1.35 for its sulfoxide. HT—C₁ gives positive reactions with ninhydrin, chlorotolidine, Pauly reagent and Barton's reagent. The reaction according to Sakaguchi and according to Ehrlich and ultraviolet fluorescence are negative.

Aminoacid analysis after acid hydrolysis gives: Lysine (1), histidine (1), aspartic acid (3), threonine (5), serine (1), glutamic acid (2), proline (2), glycine (4), alanine (2), 1/2 cystin (2), valine (1), methionine (1), isoleucine (1), leucine (2), tyrosine (1), phenylalanine (3), NH₃.

By trypsin splitting, two fragments with the same amino acid sequences as for HT—C₂ in Table 1 of Example 1 are obtained. Tr_{II} is identical for HT—C₁ and HT—C₂.

For fragment Tr_I Rs 52 (on Alox)=0.55; Rs 101 A (on cellulose)=0.86. In electrophoresis on Avicel cellulose d=0.91.

For fragment Tr_{II} Rs 52 (on Alox)=1.38; Rs 101 A (on cellulose)=1.31. In electrophoresis on Avicel cellulose d=0.94.

HT—C₂ is dissolved in 50 times its own weight of a 0.1% cysteine hydrochloride solution and kept for 1 hour at 45°C, and the

solution is then chromatographed. Apart from other products, a spot corresponding to HT—C₁ is found.

When HT—C₁ is heated with 0.5N-ammonia for 1 hour at 45°C, it is converted into HT—C₂. 70

EXAMPLE 3

When medulla carcinoma is extracted as described in Example 1, the dry residue is purified by gel filtration and the resulting product (containing 50 MRC units/mg) is subjected to a multiplicative distribution as described in Example 1, there are obtained apart from the fractions with a maximum activity K=0.6 fractions with K=2.7. The peptide HT—C₂₁ obtained therefrom is unitary in thin-layer chromatography and electrophoresis. It has the same aminoacid composition as HT—C₂, but it has no free α-amino group. It reveals in the rat an activity of about 100—200 MRC units per mg of peptide. 75 80 85

In thin-layer chromatography on Alox DO Rs 52=1.40; Rs 104=1.40; Rs 79=1.80. In the electrophoresis on Avicel cellulose d=0.56. 90

The product gives positive color reactions with ninhydrin, chlorotolidine, potassium hexacyanoferrate(II)-iron (III) chloride and diazotized sulfanilic acid amide. 95

EXAMPLE 4

When medulla carcinoma is extracted as described in Example 2, the dry residue of the extract purified by gel filtration and the product of fractions 13—21 is subjected to a multiplicative distribution, there are obtained—apart from the active fractions with K=0.19—fractions with K=4.0. The peptide HT—C₁₁ obtained therefrom proves to be unitary in thin-layer chromatography and electrophoresis. In the rat it displays an activity of about 100—200 MRC units per mg of peptide. In thin-layer chromatography on Alox DO Rs 52=1.45; Rs 104=1.60; Rs 79=1.60. In the electrophoresis on Avicel cellulose d=0.54. The product gives positive color reactions with ninhydrin, chlorotolidine, potassium hexacyanoferrate(II)-iron(III)chloride and diazotized sulfanilic acid amide. 100 105 110

EXAMPLE 5

A solution of 0.5 mg of

Cys-Gly-Asn-Leu-ser - Thr -Cys -

Met - Leu - Gly - Thr - Tyr - Thr - Gln - Asp - Phe - Asn - Lys - Phe - His - Thr - Phe - Pro - Gln - Thr - Ala - Ile - Gly - Val - Gly - Ala - Pro - NH₂ (I) [HT—C₂] in 1 ml - of an aqueous solution of 20 mg of mannitol and 4.6 mg of formic acid is filtered sterile and under aseptic conditions charged into a 5 ml-ampoule, then deep-cooled and lyophilized. 120 125

Before use the lyophilizate is dissolved in distilled water. The contents of the ampoule corresponds to about 70 MRC units. The solution is injected intramuscularly or intravenously, for example 10 to 70 MRC-units per day.

EXAMPLE 6

A suspension is prepared by dissolving 5.25 mg of ZnCl_2 and 1.05 mg of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 0.5 ml of 0.1 N hydrochloric acid, adding with stirring a solution of 1.0 mg of calcitonin M-HT-C₂ in 0.25 ml of distilled water, then 0.5 N NaOH up to pH 8.6 and filling up to 1 ml with distilled water.

The suspension corresponds to about 150 MRC-units. It can be preserved with a suitable preservative, for instance with 10 mg of benzyl alcohol.

The suspension can be used for intramuscular injection, for example 25 to 75 MRC units per day.

WHAT WE CLAIM IS:—

1. A process for the manufacture of human calcitonin wherein de-fatted medullar carcinoma of the thyroid gland or C-cell metastasis material which may have been first purified with alcohol or dilute aqueous trichloroacetic acid is extracted one or more times with a solvent system containing water and an alkanol having at most 5 carbon atoms at a pH of from about 1 to 6 and the extracted product subjected to gel chromatography using aqueous formic acid as eluant.

2. A process as claimed in claim 1 wherein the extraction is carried out using an aqueous solvent system containing acetic acid and n-butanol.

3. A process as claimed in claim 1 or claim 2 wherein the gel chromatography is effected on Sephadex G50 (as hereinbefore defined).

4. A process as claimed in claim 1 or claim 2 wherein the gel chromatography is effected on Bio Gel P6 (as hereinbefore defined).

5. A process as claimed in any of claims 1 to 4, wherein the product is separated by countercurrent distribution into the components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁.

6. A process as claimed in claim 1 carried out substantially as described in any one of Examples 1 to 4 herein.

7. Human calcitonin in the form of its components HT-C₁, HT-C₂, HT-C₁₁ and/or HT-C₂₁ or an acid addition salt or complex thereof.

8. The dotriacontapeptide amide of the formula

Cys - Gly - ASn - Leu - Ser - Thr - Cys
Met - Leu - Gly - Thr - Tyr - Thr - Gln -
Asp - Phe - Asn - Lys - Phe - His - Thr -
Phe - Pro - Gln - Thr - Ala - Ile - Gly -
Val - Gly - Ala - Pro - NH₂, or an acid
addition salt or complex thereof.

9. The dimer of the dotriacontapeptide amide of the formula

Cys-Gly-Asn-Leu-Ser-Thr - Cys
Met - Leu - Gly - Thr - Tyr - Thr - Gln -
Asp - Phe - Asn - Lys - Phe - His - Thr -
Phe - Pro - Gln - Thr - Ala - Ile - Gly -
Val - Gly - Ala - Pro - NH₂, or an acid
addition salt or complex thereof.

10. A pharmaceutical preparation containing one or more of the components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁ of human calcitonin, or a physiologically tolerable acid addition salt or complex thereof, in admixture or conjunction with a pharmaceutically acceptable carrier.

11. A pharmaceutical preparation as claimed in claim 10 substantially as described in Example 5 or Example 6 herein.

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Before use the lyophilizate is dissolved in distilled water. The contents of the ampoule corresponds to about 70 MRC units. The solution is injected intramuscularly or intravenously, for example 10 to 70 MRC-units per day.

EXAMPLE 6

A suspension is prepared by dissolving 5.25 mg of ZnCl_2 and 1.05 mg of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 0.5 ml of 0.1 N hydrochloric acid, adding with stirring a solution of 1.0 mg of calcitonin M-HT-C₂ in 0.25 ml of distilled water, then 0.5 N NaOH up to pH 8.6 and filling up to 1 ml with distilled water.

The suspension corresponds to about 150 MRC-units. It can be preserved with a suitable preservative, for instance with 10 mg of benzyl alcohol.

The suspension can be used for intramuscular injection, for example 25 to 75 MRC units per day.

WHAT WE CLAIM IS:—

1. A process for the manufacture of human calcitonin wherein de-fatted medullar carcinoma of the thyroid gland or C-cell metastasis material which may have been first purified with alcohol or dilute aqueous trichloroacetic acid is extracted one or more times with a solvent system containing water and an alkanol having at most 5 carbon atoms at a pH of from about 1 to 6 and the extracted product subjected to gel chromatography using aqueous formic acid as eluant.

2. A process as claimed in claim 1 wherein the extraction is carried out using an aqueous solvent system containing acetic acid and n-butanol.

3. A process as claimed in claim 1 or claim 2 wherein the gel chromatography is effected on Sephadex G50 (as hereinbefore defined).

4. A process as claimed in claim 1 or claim 2 wherein the gel chromatography is effected on Bio Gel P6 (as hereinbefore defined).

5. A process as claimed in any of claims 1 to 4, wherein the product is separated by countercurrent distribution into the components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁.

6. A process as claimed in claim 1 carried out substantially as described in any one of Examples 1 to 4 herein.

7. Human calcitonin in the form of its components HT-C₁, HT-C₂, HT-C₁₁ and/or HT-C₂₁ or an acid addition salt or complex thereof.

8. The dotriacontapeptide amide of the formula

Cys - Gly - ASn - Leu - Ser - Thr - Cys
Met - Leu - Gly - Thr - Tyr - Thr - Gln -
Asp - Phe - Asn - Lys - Phe - His - Thr -
Phe - Pro - Gln - Thr - Ala - Ile - Gly -
Val - Gly - Ala - Pro - NH₂, or an acid addition salt or complex thereof.

9. The dimer of the dotriacontapeptide amide of the formula

Cys-Gly-Asn-Leu-Ser-Thr - Cys
Met - Leu - Gly - Thr - Tyr - Thr - Gln -
Asp - Phe - Asn - Lys - Phe - His - Thr -
Phe - Pro - Gln - Thr - Ala - Ile - Gly -
Val - Gly - Ala - Pro - NH₂, or an acid addition salt or complex thereof.

10. A pharmaceutical preparation containing one or more of the components HT-C₁, HT-C₂, HT-C₁₁ and HT-C₂₁ of human calcitonin, or a physiologically tolerable acid addition salt or complex thereof, in admixture or conjunction with a pharmaceutically acceptable carrier.

11. A pharmaceutical preparation as claimed in claim 10 substantially as described in Example 5 or Example 6 herein.

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