Isolation and characterization of the bioactive circulating human parathyroid hormone, hPTH-1–37

Dieter Hock[a,*], Markus Mägerlein[b], Gabi Heine[a], Peter P. Ochlich[b], Wolf-Georg Forssmann[a]

[a]*Niedersächsisches Institut für Peptid-Forschung (IPF), Feodor-Lynen-Str. 31, D-30625 Hannover, Germany*
[b]*Boehringer Mannheim GmbH, Sandhofer Str. 116, D-68305 Mannheim, Germany*

Received 8 October 1996; revised version received 13 November 1996

Abstract The occurrence of hPTH-1–37 as the native bioactive circulating form of PTH-1–84 has now been obtained using a specific purification procedure for circulating parathyroid hormone, which involves a newly developed immunoenzymetric assay for N-terminally intact hPTH. In combination with two different methods of mass spectrometry, the molecular weight of the isolated immunoreactive peptide was shown to be 4401 Da, which corresponds to hPTH-1–37. Synthetic hPTH-1–37 material was tested in the chick bioassay and produced a clear-cut increase in serum calcium concentration. We conclude that hPTH-1–37 is the native bioactive fragment of hPTH-1–84 in circulation.

Key words: Parathyroid hormone; Isolation; Hemofiltrate; Dialysis; Human

1. Introduction

Parathyroid hormone (PTH) is synthesized in the chief cells of the parathyroid glands as part of the precursor molecule, prepro-PTH [1]. After cleavage of the prepro-sequence in the rough endoplasmic reticulum and Golgi apparatus, PTH is concentrated in dense-cored secretory vesicles. These vesicles fuse with the plasma membrane and PTH is released in response to decreased serum calcium levels. Under physiological conditions, secretion occurs predominantly as the intact hPTH-1–84 molecule with a molecular weight of 9425 Da [2], and a minor fraction of hPTH was shown to be metabolized prior to secretion. For human pathological parathyroid tissue, a Ca^{2+}-dependent release of quantities of N-terminal PTH fragments about 3–6 times greater than those of intact hPTH-1–84 is described [3]. The molecular forms which were shown by immunoassays or by radioactive labeling experiments to occur in the circulation [4,5] comprised mainly C-terminal and midregional fragments [6]. The generally lower concentrations of circulating N-terminal fragments were explained by peritubular uptake of N-terminal PTH by the kidney in addition to filtration [7,8] and selective uptake of N-terminal PTH fragments by bone [9]. Canterbury et al. [10] found that bovine PTH-1–84 was metabolized to N-terminal fragments by rat liver. However, given the fact that in the majority of studies bovine PTH was tested in heterologous species and taking into account the scarce information pertaining to man, the principal objective of the present investigation was to identify the major bioactive hPTH fragment in human hemofiltrate which would represent a valuable prepurified source for the large-scale extraction of smaller circulating peptides [11]. RIA and ELISA measurements [12] showed concentrations of regulatory peptides similar to those observed in plasma. Our earlier studies have shown that C-terminal hPTH fragments can be isolated from human blood filtrate [13] and for this reason, we have extensively studied the probably most important natural bioactive N-terminal molecular form [14] circulating in human blood. This present paper gives more substantial evidence that hPTH-1–37 occurs in human blood, as is revealed by isolation with mass spectrometry and immunoenzymetric assay screening.

2. Materials and methods

2.1. Purification of parathyroid hormone

Hemofiltrate was collected from patients with chronic renal failure and was obtained from a local dialysis center (Niedersächsisches Zentrum für Nephrologie, Hannoversch-Münden, Germany). During hemofiltration, a filter with a 20 000 Da cut-off was used in order to reduce the amount of filtered large molecular proteins.

The first step of purification was carried out with a modification [15] of the polypeptide isolation procedure described by Mutt [16]. After collection, hemofiltrate was immediately acidified with HCl to pH 3.5. A 1000 liter batch was diluted 1:1 (v/v) with water, adjusted to pH 2.7 by adding concentrated HCl, and stirred for 12 h after addition of 2.5 kg alginic acid. The batch was then filtered on a Büchner funnel to obtain dry alginic acid with the absorbed peptides. The alginic acid was subsequently washed with 98% (v/v) ethanol to eliminate non-peptidergic substances from the binding matrix and 0.005 N HCl. The polypeptides were then eluted with ice-cold 0.2 N HCl and lyophilized (step 1).

60 g of the extract obtained from 1000 l of hemofiltrate was resuspended in 1000 ml of loading buffer (0.01 M phosphate buffer containing 0.15 M sodium chloride, pH 7.2). The solution was filtered and applied to an affinity column (PerSeptiv, Sensor Cartridge XL, Wiesbaden, Germany) at a flow rate of 0.5 ml/min.

For the preparation of the affinity column, a polyclonal antibody specific to the N-terminal residue of hPTH was first purified on EAH Sepharose (Pharmacia, Freiburg, Germany) to which hPTH-1–34 was covalently bound. The purified antibody was immobilized using the method described by Schneider et al. [17]. In brief, the F_c region of the antibody was bound to the protein G-coated matrix of the Sensor Cartridge XL affinity column. Subsequently, the F_{ab} region was orientated with dimethyl pimelimidate (DMP) in order to achieve the optimal antibody-antigen binding for separation of the hPTH-related material. After loading with the sample, the column was washed with loading buffer in order to obtain nonspecifically bound material, as controlled by optical density measurement at 215 nm. The column was then washed with eluting buffer (0.15 M sodium chloride, pH 2.4) (step 2). The eluted immunoreactive peak was immediately transferred to a semipreparative reverse-phase (RP) C_4 column (250 Å, 20–45 μm, 20×150 mm, Parcosil, Biotek, Östringen, Germany). The column was eluted using a continuous gradient starting with 10% acetonitrile and 0.1% TFA to 80% acetonitrile and 0.1% TFA (step 3). Fractions were identified by means of a two-site immunoenzymetric assay (IEMA) for N-terminal intact hPTH. The immunoreactive fractions were pooled, diluted with water and directly applied to the next chromatographic step, performed with an analytical RP C_{18}

*Corresponding author. Institut für Anatomie und Zellbiologie I, Im Neuenheimer Feld 307, D-69120 Heidelberg, Germany.
Fax: (49) (6221) 54 4951.
E-mail: Hock@novsrv1.pio1.uni-Heidelberg.de

PII S0014-5793(96)01390-7

column (300 Å, 5 μm, 4.6×250 mm, Vydac, Hesperia, CA, USA), using a gradient from 10% acetonitrile, 0.1% TFA to 60% acetonitrile, 0.1% TFA (Step 4). After this purification, all the fractions were determined by means of IEMA.

2.2. Mass spectrometry

Mass determination of fraction 24, which represents the highest activity in the IEMA, was carried out by two different methods. First, a combination of HPLC and mass spectrometry (LC-MS mode) was performed. LC/MS was conducted using an ABI 140A pump (Weiterstadt, Germany) to provide gradients with a flow rate of 20 μl/min. A microbore RP C_{18} YMC ODS-AQ (250×1 ID, 120 Å, 3 μm, YMC, Wilmington, NC, USA) column was used and the gradient was 10% B to 70% B in 30 min (2%/min). Solvent A was 0.05% aqueous TFA and solvent B was water/acetonitrile/TFA (20:80:0.06). The column effluent was directed to a variable wavelength detector (750 A, ABI, Weiterstadt, Germany) set to monitor absorbance at 210 nm, and thereafter into a Sciex API III triple quadrupole mass spectrometer (Perkin Elmer, Sciex Instruments, Thornhill, Ont., Canada). The interface sprayer was operated at a positive potential of 5 kV. Positively charged ions were sampled in the analyzer region of the mass spectrometer through a 100 μm orifice with an applied voltage of 60–100 V. Full scan single mass spectra were obtained by scanning quadrupole-1 from 300 to 2400 m/z with a step size of 0.5 Da. A mixture of polypropylene glycols was used for mass calibration in the positive-ion module. Data were put into an Apple Macintosh computer and processed using the data analysis program MacSpec 3.1 (Sciex).

For a second set of measurements, a matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometer (LaserTec time-of-flight mass spectrometer, Vestec, Houston, TX, USA) was used in the positive-ion mode of detection. Samples were prepared by mixing 1.5 μl of the fraction possessing the highest immunological activity with 1.5 μl of saturated solution of α-cyano-4-hydroxycinnamic acid (as matrix) and applying the combined 3 μl to a stainless steel insertion probe tip. Preparations were allowed to air-dry before insertion into the vacuum system of the mass spectrometer. The time-of-flight spectra were generated by a signal averaging 120 laser shots into a single spectrum and its transfer to an IBM-compatible personal computer. Spectra were calibrated using saturated α-cyano-4-hydroxycinnamic acid as the matrix ion, and ubiquitin (MH^+ 8565.9, MH^{2+} 4282.95) as the protein standard.

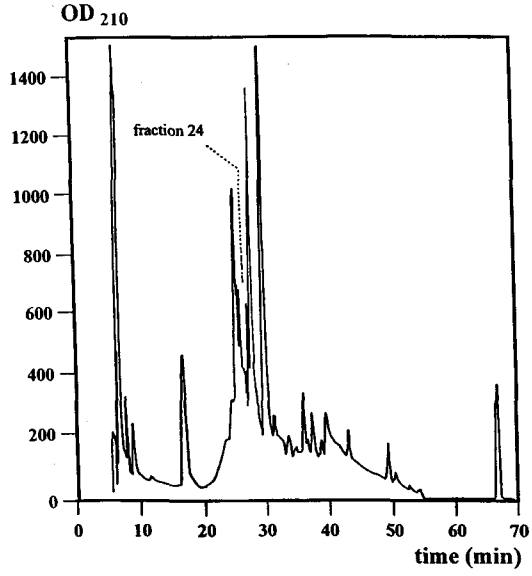

Fig. 1. Analytical reverse-phase HPLC profile on a Vydac-C_{18} column (250×4.6 mm ID). Solvent system: A: 0.1% TFA, B: 0.1% TFA in aqueous acetonitrile; flow rate 0.7 ml/min. Absorbance was measured at 210 nm. Crude extract of hemofiltrate purified by affinity chromatography and applied twice to a semipreparative reverse-phase column. IR-hPTH is found in fraction 24 only (see Fig. 2).

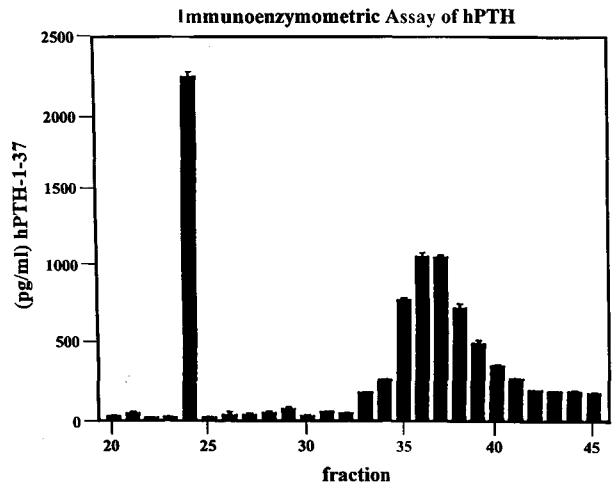

Fig. 2. Immunoenzymetric assay (IEMA) from aliquots of the analytical RP HPLC fractions. The immunoreactivity of fraction 24 represents hPTH-1–37. The minor activity at the end of the chromatography derived from C-terminal-extended PTH fragments.

2.3. Immunoenzymetric assay (IEMA)

The IEMA procedure [18] was carried out in duplicate in microtiter plates (Maxisorb, Nunc, Wiesbaden, Germany). The wells were coated overnight with 0.5 μg/well of a monoclonal antibody. After the wells were washed, blocking buffer (0.05 M sodium carbonate buffer pH 9.4 with 1% gelatin) was added for 2 h to prevent nonspecific binding. Thereafter, 100 μl sample or standard was added for 1 h. In the next step, 100 μl of the second antibody (a polyclonal antibody, purified by affinity chromatography; dilution 1:1000) was added for 1 h. This antibody was marked with 100 μl AffiniPure anti-rabbit IgG-POD at a dilution of 1:5000 (Dianova, Hamburg, Germany). The final step was the reaction with 100 μl substrate buffer (Boehringer Mannheim GmbH, Mannheim, Germany) containing 0.2 M ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] [6], Boehringer Mannheim, Mannheim, Germany) for 30 min and absorbance was measured at 405 nm. Between the various steps, extensive washing of the wells with buffer (PBS 0.015 M, 0.05% Tween 20) was performed.

2.4. Bioassay

The biological activity of hPTH-1–37 was tested in the chick bioassay [19] in terms of its ability to increase the serum calcium concentration. Three different doses (1.0, 2.5 and 6.25 μg/animal) were injected into the wing vein of 10-day-old chicks. After 60 min, the animals were anesthetized and bled for analysis of serum calcium by means of atomic absorption spectroscopy (Atomic Absorption Spectrometer M 2100, Perkin Elmer, Munich, Germany).

3. Results and discussion

Parathyroid hormone is rapidly metabolized, mainly by the liver and kidney, to smaller peptides of mostly unknown biological activity. Generation of circulating biologically active N-terminal PTH has been suggested on the basis of certain observations in vitro [20,21], and what are believed to be N-terminal fragments may be detectable in blood under pathological circumstances in vivo [3]. The determination of N-terminal fragments of PTH in the blood was carried out using immunological [22,23] and chromatographic methods (such as retention time) [24], or a combination of both [25]. These methods, however, do not allow a definitive conclusion about the exact molecular form which is characterized by a distinct molecular weight and related amino acid sequence.

Taking the above findings into account, we started an extraction procedure with a readily accessible equivalent of hu-

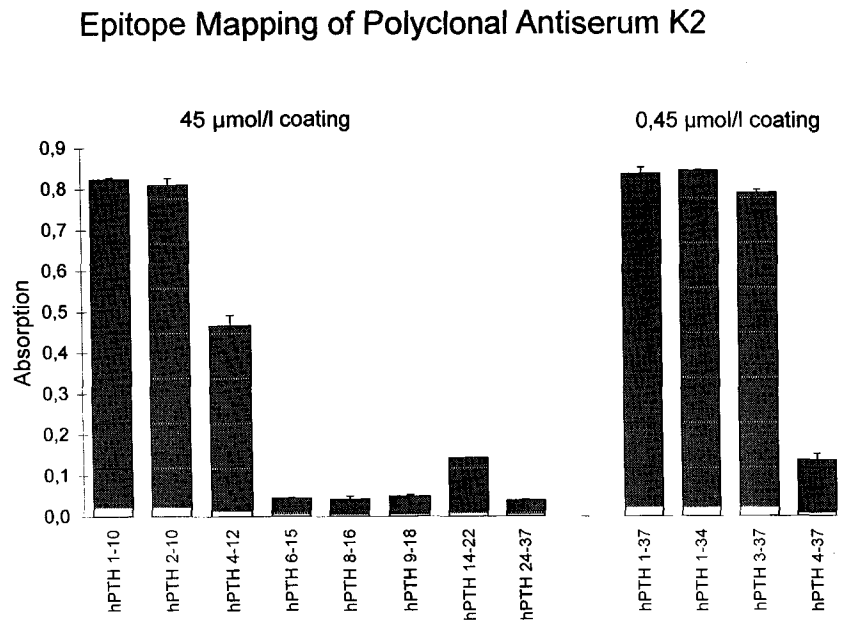

Fig. 3. Epitope mapping of the polyclonal antibody was used for the affinity chromatography.

man plasma, namely hemofiltrate, which contains mostly plasma peptides $< 20\,000$ Da. This starting material was first treated by ion-exchange absorption on alginic acid in organic and aqueous solution (step 1) and elution of the crude peptide extract. Affinity chromatography (step 2) and two reverse-phase HPLC separations on C_4 and C_{18} supports (steps 3 and 4) yielded a purification to identify the N-terminal hPTH molecular form. Aliquots from the analytical RP separation (Fig. 1) were measured by means of IEMA (Fig. 2) to detect the N-terminal parathyroid hormone [18]. This assay detects hPTH-1–84 and fragments of this peptide if the N-terminus is intact (Fig. 3). The main activity eluted in fraction 24 which was found to be a bioactive (Fig. 4) 4404 Da (Figs. 5 and 6) peptide. The remaining activity corresponded to the intact hPTH-1–84 on this RP HPLC column.

These findings are in general agreement with those reported by Schettler et al. [26] who analyzed N-terminal hPTH fragments by means of RP HPLC and RIA from human plasma extracts from healthy individuals, osteoporotic, hyperparathyroid, and pseudohyperparathyroid patients. The pattern of the different fragments was considered to be disease-specific in their proportion. One peak eluting consistently in all cases was supposed to represent hPTH-1–38 [26]. The results obtained in the present study are in agreement with those of earlier investigations of our group [12] demonstrating the occurrence of two C-terminal fragments, hPTH-38–84 and hPTH-38–54 in hemofiltrate which clearly demonstrate the crucial cleavage site to result in hPTH-1–37 at the N-terminal portion. The possibility of an enzymatic cleavage of hPTH-1–84 was investigated by Botti et al. [27] using acid endopeptidase of a kidney cortex plasma membrane preparation. This cleavage obtained by a bovine endopeptidase of the kidney may, however, not represent a metabolic pathway leading to the circulating hPTH form. In their experiments, hydrolysis of PTH-1–84 generated accumulating levels of C-terminal fragments comprising the residues 35 and 38 as demonstrated by microsequencing the cleaved fragments. However, Bringhurst et al. [28] were able to demonstrate the in vivo existence of N-terminal bioactive bPTH fragments. Nevertheless, these authors concluded that a cleavage may occur in position 30–35 of intact PTH-1–84 by endopeptidases of the liver and kidney, generating both N- and C-terminal fragments, with the N-terminal fragment(s) tending to accumulate in the kidneys. In this context, it should be emphasized that hemofiltrate from patients with chronic renal failure was used in our experiments, i.e. impaired renal clearance of PTH fragments as a cause of higher endogenous levels is likely to occur [29].

To check for the generation of artefacts during storage and purification of the hemofiltrate, hPTH-1–84 and N-terminal fragments 1–32 to 1–38 (except for 1–36) were added to hemofiltrate for 24 h. There was no degradation of the tested peptides, as judged by RP HPLC. In view of the considerable loss of material due to the hydrophobicity of hPTH-1–37, it is not possible to estimate the physiological plasma concentra-

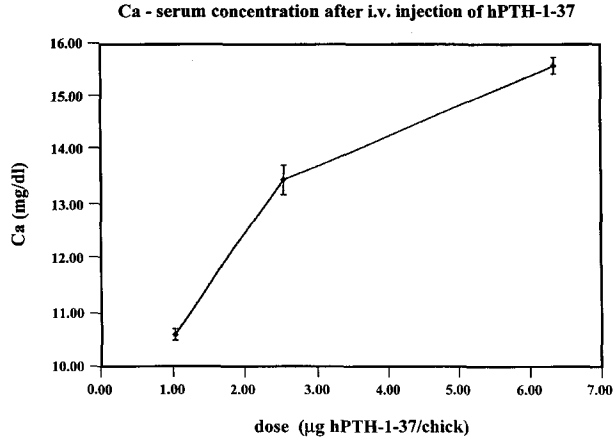

Fig. 4. Biological activity of hPTH-1–37 (1.0, 2.5 and 6.25 µg/animal) after *intravenous* injection into a chick. Changes in Ca plasma concentration were measured (atomic absorption spectroscopy) 1 h after administration. Values are given as mean ± SEM, $n = 6$.

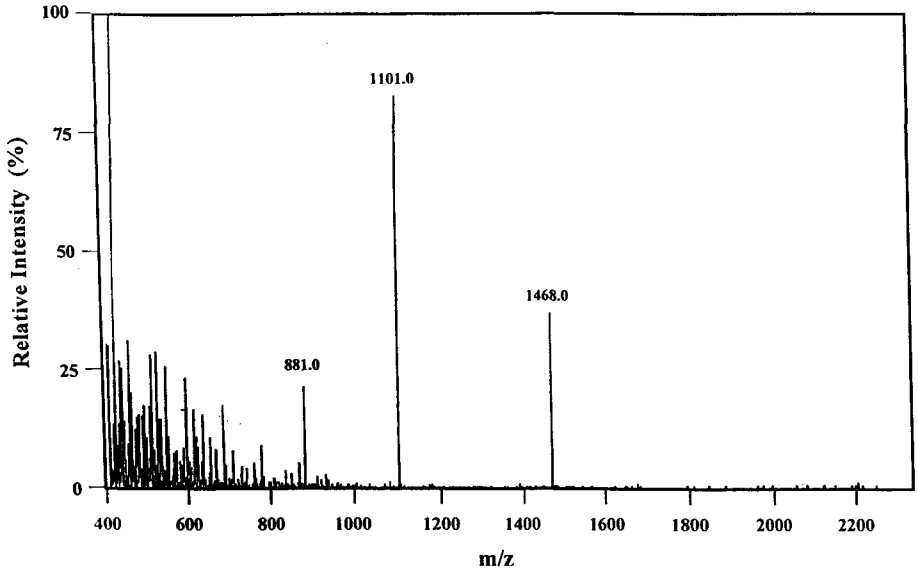

Fig. 5. LC-MS of immunoreactive fraction 24, using a microbore RP-C_{18} column (YMC, 250×1 mm ID) and a Sciex API-III ion-spray mass detector. The prominent ion at m/z 1101 is charged fourfold.

tions of this peptide in vivo. Bringhurst et al. [28] investigated the fate of N-terminal fragments. Using bovine [^{35}S]PTH-1–84 in combination with high resolution chromatographic techniques sensitive to 10^{-13} M PTH peptides, there was no evidence of generation of circulating N-terminal PTH fragments. It was shown that the N-terminal end of PTH was rapidly degraded in situ by the liver, but that both liver and kidneys nevertheless contained low levels of N-terminal fragments. It was concluded that the peripheral metabolism of PTH in normal rats does not lead to circulating N-terminal fragments of the hormone. In this study, however, bovine PTH was used and the tests were carried out in rats, being a heterologous species. Similarly, a number of studies designed to clarify the metabolic fate of hPTH-1–84 [10,30–35] were performed in heterologous systems which may inherently result in unnatural metabolic kinetics.

From the literature available so far it may be postulated that hPTH-1–37 is cleaved to hPTH-1–34. This hypothesis was, however, not confirmed by our findings since we could not identify any fragment which may relate to hPTH-1–34.

References

[1] Kemper, B., Habener, J.F. and Mulligan, R.C. (1974) Proc. Natl. Acad. Sci. USA 71, 3731–3735.
[2] Hendy, G.N., Kronenberg, H.M., Potts, J.R., Jr. and Rich, A. (1981) Proc. Natl. Acad. Sci. USA 78, 7365–7369.
[3] Fuleihan El-Hajj, G., Chen, C.J., Rivkees, S.A., Marynick, S.P., Stock, J., Palotta, J.A. and Brown, E.M. (1989) J. Clin. Endocrinol. Metab. 69, 860–867.
[4] Bringhurst, F.R., Stern, A.M., Yotts, M., Muzsatin, M., Segre, G.V. and Potts, J.T., Jr. (1988) Am. J. Physiol. 255, E886–E893.

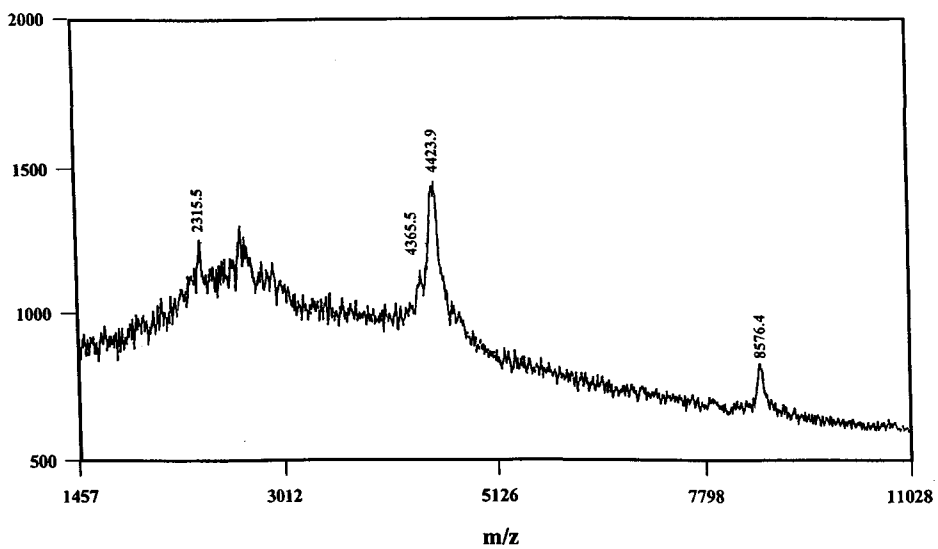

Fig. 6. Matrix-assisted laser-induced desorption/ionization mass spectrometry (MALDI-MS) carried out with a LaserTec time-of-flight mass spectrometer (Vestec) on fraction 24 from the analytical RP HPLC run. The spectra were produced by a signal averaging 120 laser shots. α-Cyano-4-hydroxycinnamic acid was used as the matrix. Note the exact molecular mass of Na-hPTH-1–37 is determined (4423.9).

[5] Segre, G.V., Habener, J.F., Powell, D., Tregear, G.W. and Potts, J.T., Jr. (1972) J. Clin. Invest. 51, 3163–3172.
[6] Goltzmann, D., Gomolin, H., Edlean, A., Wexler, M. and Meakins, J. (1984) J. Clin. Endocrinol. Metab. 58, 70–75.
[7] Hruska, K.A., Kopelman, R., Rotherford, W.E., Klahr, S. and Slatopolsky, E. (1975) J. Clin. Invest. 56, 39–48.
[8] Hruska, K.A., Martin, K., Mennes, P., Greenwalt, A., Anderson, C., Klahr, S. and Slatopolsky, E. (1977) J. Clin. Invest. 60, 501–510.
[9] Martin, K.J., Finch, J.L., Hruska, K. and Slatopolsky, E. (1987) Kidney Int. 31. 937–940.
[10] Canterbury, J.M., Bricker, G.S., Levey, G.S., Kozlovskis, P.L., Riuz, E., Zull, J.E. and Reiss, E. (1975) J. Clin. Invest. 55, 1245–1253.
[11] Forssmann, W.G., Schulz-Knappe, P., Meyer, M., Adermann, K., Forssmann, K., Hock, D. and Aoki, A. (1993) in: Peptide Chemistry (Yanaihara, N., Ed.), pp. 553–557, Escom, Leiden.
[12] Schepky, A.G., Bensch, K.W., Schulz-Knappe, P. and Forssmann, W.G. (1994) Biomed. Chromatogr. 8, 90–94.
[13] Forssmann, W.G., Herbst, F., Schulz-Knappe, P., Adermann, K. and Gagelmann, M. (1989) Eur. Patentschrift, 0 497 915 B1.
[14] Marx, U.C., Austermann, S., Bayer, P., Adermann, K., Ejchart, A., Sticht, W., Schmid, F.X. Jaenicke, R., Forssmann, W.G. and Rŝsch, P. (1995) J. Biol. Chem. 270, 15194–15202.
[15] Hock, D., Schriek, U., Fey, E., Forssmann, W.G. and Mutt, V. (1987) J. Chromatogr. 397, 347–353.
[16] Mutt, V., (1959) Ark. Kemi 15, 69–74.
[17] Schneider, C., Newman, A.R., Sutherland, D.R., Asser, U. and Greaves, M.F. (1982) J. Biol. Chem. 257, 10766–10769.
[18] Mägerlein, M., Hock, D., Adermann, K., Muth, R., Neidlein, R., Forssmann, W.G. and Strein, K. (1995) Calcif. Tissue 56, 193.
[19] Parsons, J.A., Reit, B. and Robinson, C.J. (1973) Endocrinology 92, 454–462.
[20] Fluek, J.A., DiBella, F.P., Edis, J.A., Kehrwald, I.M. and Arnaud, C.D. (1977) J. Clin. Invest. 60, 1367–1375.
[21] Hanley, D.A., Takatsuki, K., Sultan, J.M., Schneider, A.B. and Sherwood, L.M. (1978) J. Clin. Invest. 62, 1247–1254.
[22] Roos, B.A., Lindall, A.W. and Aron, D.C. (1981) J. Clin. Endocrinol. Metab. 53, 709–721.
[23] D'Amour, P., Palardy, J., Bahsali, G., Malette, L.E., DeLean, A. and Lepag, R. (1992) J. Clin. Endocrinol. Metab. 74, 525–532.
[24] Zull, J.E. and Chuang, J. (1985) J. Biol. Chem. 260, 1608–1613.
[25] Bringhurst, F.R., Stern, A.M., Yotts, M., Mizrahi, N., Segre, G.V. and Potts, J.T., Jr. (1989) J. Endocrinol. 122, 237–245.
[26] Schettler, T., Aufm'Kolk, B., Atkinson, M.J., Radeke, H., Enters, C. and Hesch, R.D. (1984) Acta Endocrinol. 107, 60–69.
[27] Botti, Jr., R.E., Heath, E., Frelinger, A.L., Chuang, J., Roos, B.A. and Zull, J.E. (1981) J. Biol. Chem. 256, 11483–11488.
[28] Bringhurst, F.R., Stern, A.M., Yotts, M., Muzsatin, M., Segre, G.V. and Potts, J.T., Jr. (1988) Am. J. Physiol. 255, E886–E893.
[29] Cohen Solal, M.E., Sebert, J.L., Boudailliez, B., Marie, A., Moriniere, M.P., Gueris, J., Bouillon, R. and Fournier, A. (1991) J. Clin. Endocrinol. 73, 516–524.
[30] Segre, G.V., Perkins, L.A. and Witters, (1981) J. Clin. Invest. 67, 449–457.
[31] D'Amour, P., Segre, G.V., Roth, S.I. and Potts, J.T., Jr. (1979) J. Clin. Invest. 63, 89–98.
[32] Martin, K.J., Freitag, J.J., Conrades, M.B. and Hruska, K.A. (1978) J. Clin. Invest. 256–261.
[33] Freitag, J.J., Martin, K.J., Conrades, M.B. and Slatopolsky, E. (1979) Endocrinology 104, 510–516.
[34] Pillai, S. and Zull, J.E. (1986) Am. Soc. Biol. Chemists 261, 1419–1423.
[35] MacGregor, R.R., Hamilton, J.W., Kent, G.N., Shofstaff, R.E. and Cohn, D.V. (1979) J. Biol. Chem. 254, 4428–4433.