Journal of Chromatography, 276 (1983) 55—68
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1722

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF HUMAN PARATHYROID HORMONE IN REFERENCE STANDARDS, PARATHYROID TISSUE AND BIOLOGICAL FLUIDS

JOAN M. ZANELLI*, J.C. KENT and B. RAFFERTY

National Institute for Biological Standards and Control, Holly Hill, London NW3 6RB (Great Britain)

R.A. NISSENSON

Veterans Administration Hospital, 4150 Clement Street, San Francisco, CA 94121 (U.S.A.)

and

E.C. NICE, M.W. CAPP and M.J. O'HARE

Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Sutton, Surrey SM2 5PX (Great Britain)

(First received December 30th, 1982; revised manuscript received February 28th, 1983)

SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used to fractionate human parathyroid hormone (hPTH) from a variety of natural sources and to compare it with synthetic hPTH and hPTH fragments. Multiple radioimmunoassay systems for amino, mid and carboxyl regions of hPTH were used to monitor various preparations of hPTH previously prepared by conventional methods and ampouled in nanogram amounts for reference standard and reagent purposes. Results confirmed that they were free of detectable cleavage products, but showed that the intact hPTH comprised three or four closely associated components. A similar pattern of heterogeneity was obtained when hPTH was extracted from stored human parathyroid adenomata by a simple rapid HPLC bulk fractionation method. Comparison with synthetic 1—84 hPTH and modification of sample handling to minimize oxidative conditions, indicate that some of these components are probably intermediate oxidation products. A number of less hydrophobic components, with carboxyl region immunoreactivities, were obtained from the individual adenoma samples, human parathyroid cyst fluid, ampouled samples of human adenoma tissue culture medium, and secondary hyperparathyroid plasma ultrafiltrate when they were fractionated by RP-HPLC. The results strongly suggest that the biological degradation of hPTH is more complex than generally believed, and that RP-HPLC offers a new dimension in its analysis.

0378-4347/83/$03.00

INTRODUCTION

Reversed-phase high-performance liquid chromatographic methods (RP-HPLC) have now been used to separate a wide variety of polypeptide [1,2] and protein hormones [3,4]. We have recently applied these techniques, which are based on the interaction of hydrophobic residues with alkylsilane-bonded silicas of various carbon loadings, chain-lengths and pore sizes, to the characterization of bovine parathyroid hormone (bPTH) [5]. The results based on UV absorbance, endogenous tryptophan fluorescence chromatographic profiles, region specific radioimmunoassays (RIA) and in vivo bioassay showed that high yields of biologically active material could be readily separated from oxidation and other degradation products using an octadecylsilane-bonded (C-18) 8-nm pore-size packing. These results suggested that similar methods coupled with region-specific RIA might provide a high-resolution, high-recovery system for the analysis of human parathyroid hormone (hPTH) and associated materials of biological origin.

This paper reports the fractionation, by RP-HPLC, of components in a variety of preparations of hPTH, a polypeptide of 84 residues. The preparations included the World Health Organisation (WHO) International Reference Preparation which was prepared and purified by conventional lengthy extraction and chromatographic procedures, other ampouled preparations similar to those used as working standards in immuno-assay systems, as well as materials extracted from individual parathyroid adenomata and related biological fluids and processed solely by rapid HPLC methods.

Three ampouled preparations of extracted hormone and the two ampouled samples of biological fluids analysed here have been included in a recently completed international collaborative study, organised on behalf of the WHO, to characterize and calibrate by RIA and in vitro bioassay the First International Reference Preparation of Parathyroid Hormone, Human, for Immunoassay (IRP hPTH) [6].

EXPERIMENTAL

The five ampouled preparations included in this study have been described in detail in the report of the WHO international collaborative study [6]. In summary the preparations comprised two groups:

1. Preparations derived from extracts of pooled human parathyroid adenomata and hyperplastic tissues

NIBSC research reagent, ampoule code 78/551. Unpurified hPTH was extracted from pooled lyophilized tissues using the urea—HCl procedure [7] and precipitated with trichloroacetic acid (TCA). The extract prepared and donated to NIBSC by Drs. C. Arnaud and B. Brewer, was estimated to be between 2% and 5% pure. Ampoules were estimated to contain the equivalent of approximately 3.6 pmole (36 ng) of hPTH.

NIBSC Research Standard hPTH for immunoassay, ampoule code 75/549. Material was extracted from pooled fresh frozen tissues by the phenol method [8], precipitated with TCA and partially purified by gel chromatography. The

material, prepared and donated to NIBSC by Drs. J.S. Woodhead and M. Peacock, was estimated to be approximately 10% pure. Ampoules contained the equivalent of approximately 2.5 pmole (25 ng) of hPTH [9].

International Reference Preparation for Parathyroid Hormone, Human, for Immunoassay (IRP hPTH), ampoule code 79/500. Material was extracted from pooled lyophilized tissue by the urea—HCl method [7] and precipitated with TCA. The TCA hPTH was purified and re-purified using gel chromatography and ion-exchange chromatography; the final product, donated to WHO by Drs. C.D. Arnaud and B. Brewer, was stated to be 95% pure. Ampoules were estimated to contain approximately 10 pmole (100 ng) of hPTH [6].

2. *Preparations derived from biological fluids*

Tissue culture hPTH. The culture medium in which human parathyroid adenomata had been maintained in culture for 6—9 days was fractionated by gel chromatography; "peak 2" covering the elution region associated with bPTH was pooled as described by Dorn and Montz [10]. A portion of the fluid, donated to NIBSC by Drs. R. Montz and G. Dorn, was diluted and freeze-dried in ampoules codes 78/616. Estimates of the content of immunoreactive hPTH varied from 2.6—146 mI.U. per ampoule depending on the immunoassay system used [6].

Plasma filtrate secondary hyperparathyroid hPTH. Plasma ultrafiltrate obtained during haemodialysis of one patient with hyperparathyroidism secondary to renal failure [11], was donated to NIBSC by Professor R. Ziegler and Dr. H. Minne. One-ml aliquots of this fluid were freeze-dried in ampoules coded 78/618. Estimates of the content of immunoreactive hPTH varied from 0.5—166 mI.U. per ampoule depending on the immunoassay systems used [6].

Ampouling

Ampouling was carried out according to procedures used for WHO International Biological Standards described by the WHO Expert Committee on Biological Standardization [12]. The preparations of extracted hPTH, ampoule code numbers 78/551, 75/549 and 79/500 contained 250 μg of human albumin, free of peptidase activity [13] as carrier, and 5 mg lactose.

Materials

Other materials used in this study included pepstatin A (Sigma, Poole, Great Britain), synthetic human calcitonin (Ciba-Geigy, Basle, Switzerland); synthetic peptide fragments of hPTH, sequences 1—34, 28—48, 44—68 and 53—84 (Bachem Fine Chemicals, Torrance, CA, U.S.A., or Uniscience, Cambridge, Great Britain); highly purified bPTH preparations characterized and described previously [6], and cathepsin products 1—30 and 37—84 of bPTH, prepared and characterized by Dr. J. Morrissey (St. Louis, MO, U.S.A.) according to published procedures [14]. Synthetic 1—84 hPTH (Asp^{76}) was generously donated to us by Dr. S. Sakakibara (Protein Research Foundation, Osaka, Japan). Details of synthesis, purification and characterization are described elsewhere [15]. Individual human parathyroid adenomata were generously provided by Drs. I. Marschner and W.G. Wood (Munich, G.F.R.); fluid aspirated from a parathyroid cyst was kindly made available by Drs. R. Ardaillou and D. Raymond (Paris, France).

High-performance liquid chromatography

The systems used in this study have been described previously in detail [1,2,5]. Briefly, polypeptides were separated on 150 mm × 4.6 mm I.D. columns, slurry packed with ODS-Hypersil (8 nm pore size, 5 μm particle size, Shandon Southern, Runcorn, Great Britain), using a primary solvent of 0.155 *M* sodium chloride—0.01 *M* hydrochloric acid (pH 2.1) with acetonitrile (Rathburn Chemicals, Walkerburn, Great Britain) as secondary solvent. Peptides were dissolved in the aqueous primary solvent for injection. The initial conditions consisted of a 2.5-min loading phase with primary (aqueous) solvent and a short rapid gradient step to 10% acetonitrile over the next 2.5 min. The peptides were then routinely eluted using a linear gradient of 10—60% acetonitrile over the next 67 min (the rate of change was 0.75% acetonitrile per min) at a constant flow-rate of 1 ml/min and a temperature of 45°C, with an Altex 324-40 chromatograph. For increased resolution of materials eluting close to intact hPTH 1—84, a slow gradient of 24—44% acetonitrile over 60 min (i.e. 0.33%/min) was used. The eluate was monitored for UV absorbance at 215 nm (LDC Spectromonitor III) and for endogenous fluorescence of tryptophan-containing peptides (225/340 nm, Schoeffel FS-970).

In all cases fractions were collected at 0.2-, 0.5- or 1-min intervals into polypropylene microcentrifuge tubes (Sarstedt U.K., Leicester, Great Britain), or

TABLE I

RIA SYSTEMS USED FOR hPTH DETERMINATIONS

Antiserum	Tracer type	Assay standard	Final antiserum dilution	Incubation time	Non-specific binding (% total)	Specific binding (% total)
I + N	^{125}I bPTH (1—84)	hPTH (1—84)	1:300,000	1 day	7	38
			1:500,000	3 + 3 days	7	29
C + I	^{125}I bPTH (1—84)	hPTH (1—84)	1:300,000	1 day	10	35
			1:500,000	3 + 3 days	8	27
N	^{125}I hPTH (1—34)	hPTH (1—34)	1:300,000	1 day	3	31
		hPTH (1—84)	1:600,000	3 + 3 days	3	22
M	^{125}I bPTH (1—84)	hPTH (44—68)	1:26,000	1 day	8	36
C	^{125}I bPTH (1—84)	hPTH (53—84)	1:600,000	1 day	8	27

*Molar ratio at 50% displacement.
**ND = Not done.
***Indicates non-parallelism of displacement curves.
§No displacement at >10-fold molar excess.

into glass tubes. For collection of pmole quantities of hPTH for immunoassay and/or bioassay, tubes containing 10 μl of a solution of bovine serum albumin (1 mg/ml Fraction V RIA grade crystalline, Sigma) were used. All eluate fractions were rapidly frozen on dry ice, freeze-dried to remove acetonitrile and stored at −40°C.

For bulk fractionation of individual adenomata and parathyroid cyst fluid prior to analytical HPLC as described above, a mini-column (80 mm × 4.6 mm I.D.) tap-packed with Partisil 10 ODS (Whatman, Maidstone, Great Britain) was used. Between 0.5 and 2 g of tissue (which had been stored at −70°C) was homogenised (UltraTurrax, Janke and Kunkel, Staufen, G.F.R.) over ice in 10 ml/g of 0.155 *M* sodium chloride—0.01 *M* hydrochloric acid (pH 2.1) readjusted to pH 2.1 after homogenisation and then centrifuged at 105,000 *g* for 30 min. In some experiments 20 μg/ml pepstatin and 5 μg/ml human calcitonin (hCT) were added to the homogenate as an inhibitor of proteolysis and an internal chromatography marker, respectively. The supernatant containing acid-soluble proteins and peptides was then trace enriched onto a mini-column, the retained materials washed with 5 ml of acid saline and polypeptides eluted by stepwise additions of 5 ml each of 20% and 60% acetonitrile in 0.155 *M* sodium chloride—0.01 *M* hydrochloric acid (pH 2.1) [2].

The acetonitrile-containing eluates were immediately subjected to analytical

Detection limit ($M \cdot 10^{-9}$)	50% Displacement ($M \cdot 10^{-9}$)	Relative affinity for*:				Comments
		hPTH (1—84)	hPTH (1—34)	hPTH (44—68)	hPTH (53—84)	
0.1	0.9	1	ND**	§	ND	Predominantly Intact and N-region hPTH
0.05	0.2	1	0.12***	ND	§	
0.1	0.6	1	ND	§	ND	Predominantly C-region and Intact hPTH
0.05	0.2	1	0.07	ND	3.6	
0.3	1.6	ND	1	ND	ND	N-region: recognizes Intact hPTH on an equimolar basis
0.06	0.6	1	1	§	§	
0.04	0.2	0.37	§	1	§	Mid-region: some recognition of Intact hPTH
0.05	0.3	0.08	0.01	§	1	Predominantly C-region, poor recognition of Intact hPTH

fractionation as described above, after removal of the organic modifier by evaporation under nitrogen. The entire process from thawing and homogenisation of solid tissues to collecting and freezing of fractions from analytical HPLC took less than 2.5 hours. An identical procedure was used for parathyroid cyst fluid, except that the homogenisation step was omitted.

Immunoassay

Freeze-dried residues from HPLC and analytical fractionation were routinely dissolved in 0.25—0.5 ml of acetic acid (0.001—0.1 *M*) and aliquots diluted in assay buffer appropriate to the immunoassay system to be used for measurement of immunoreactive hPTH, or for localisation of the hCT internal marker. Aliquots were routinely assayed undiluted or at low dilution in order to screen for non-specific interference or for low concentrations of immunoreactivity, and at multiple further dilutions for measurement of high concentrations of eluted peptides. Repeat assays were carried out as required.

Five RIA systems, with different recognition characteristics for intact (I) hPTH and for the amino (N), mid and carboxyl (C) regions of the hPTH molecule were used. Four were heterologous reagent systems based on antisera raised to bPTH, radioiodinated bPTH as tracer and hPTH as standard. In addition to antiserum Burroughs Wellcome 211/32, antisera 1127/21 and 266/5 resulting from collaborative studies with Wellcome Research Laboratories (Beckenham, Great Britain) were used [16]. The fifth RIA was an homologous system in which the synthetic h1—34 PTH peptide had been used as immunogen to raise antiserum and radioiodinated as tracer. (Antiserum G-017 was generously provided by Drs. B. Moukhtar and C. Desplan, Paris, France.) Other details of reagents and methodology are as described elsewhere [17] except that concentrations of reagents were increased and separation of bound and free tracer modified as required for the more rapid one day RIA systems.

The comparison of cross-reactivities with I, N-, mid- and C-region hPTH peptides are summarized in Table I.

RIA for hCT was modified from a published method [18] and used antiserum Burroughs Wellcome 824/7.

RESULTS

With an octadecylsilane (C-18) packing the highly purified native bPTH 1—84, the synthetic hPTH 1—84 and the marker peptide, synthetic hCT, can readily be separated using a linear gradient of acetonitrile in pH 2.1 isotonic saline as shown in the chromatogram in Fig. 1. The retention time for the hCT marker was 42 min, with bPTH 1—84 eluting a minute later at 43 min, and hPTH 1—84 eluting slightly more than a minute earlier than hCT at 40.5 min. The gradient system also gives a clear separation of the synthetic hPTH peptide fragments, 1—34, 28—48, 44—68 and 53—84 with retention times of 39, 33, 19.8 and 26.5 min, respectively (the relative elution positions for these synthetic peptides are marked on Fig. 1 for comparison). The precision with which the retention times of individual synthetic peptides could be reproduced on different occasions and with different columns of the same dimensions was ±1 min. Nevertheless, because the biological preparations

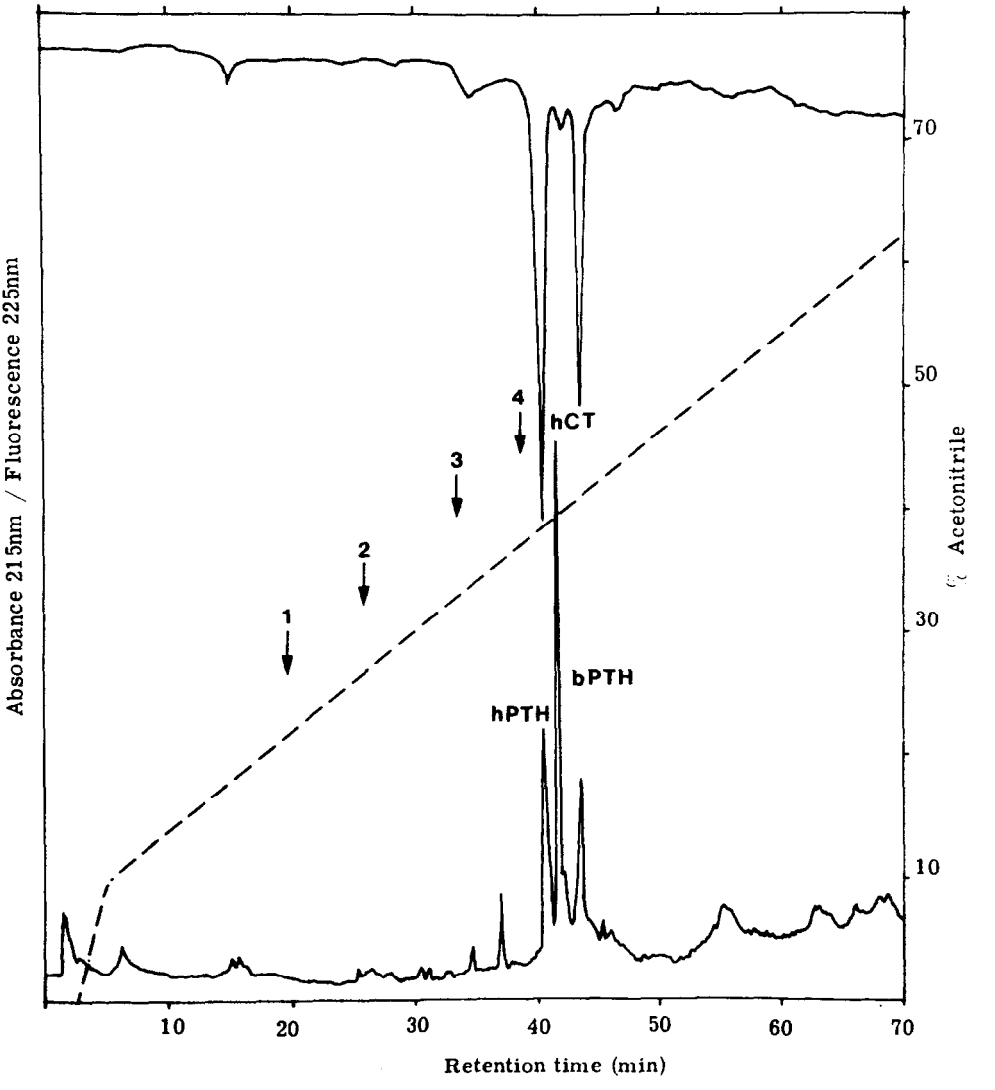

Fig. 1. UV absorbance (215 nm) (lower trace) and endogenous tryptophan fluorescence (225/340 nm) (upper trace), chromatograms of synthetic hPTH (Asp^{76}), the marker peptide synthetic hCT and highly purified native bPTH. The acetonitrile gradient as used throughout all studies in this report is shown (— — —). Arrows indicate the characteristic retention times for the synthetic peptide fragments of hPTH, in order of elution, as described in the text: (1) 44—68, (2) 53—84, (3) 28—48 and (4) 1—34.

analysed included samples with pmole quantities of hPTH which could not be detected directly by UV absorbance or fluorescence, hCT was included as an internal peptide marker in all such samples. hCT was particularly suitable for this purpose as it elutes close to the main regions associated with bPTH or hPTH and it can be localized to individual tubes by RIA as it does not cross-react in immunoassay systems for hPTH.

Ampouled preparations containing 3%, 10% and 95% pure extracted hPTH, as detailed in Experimental, and originally prepared by conventional proce-

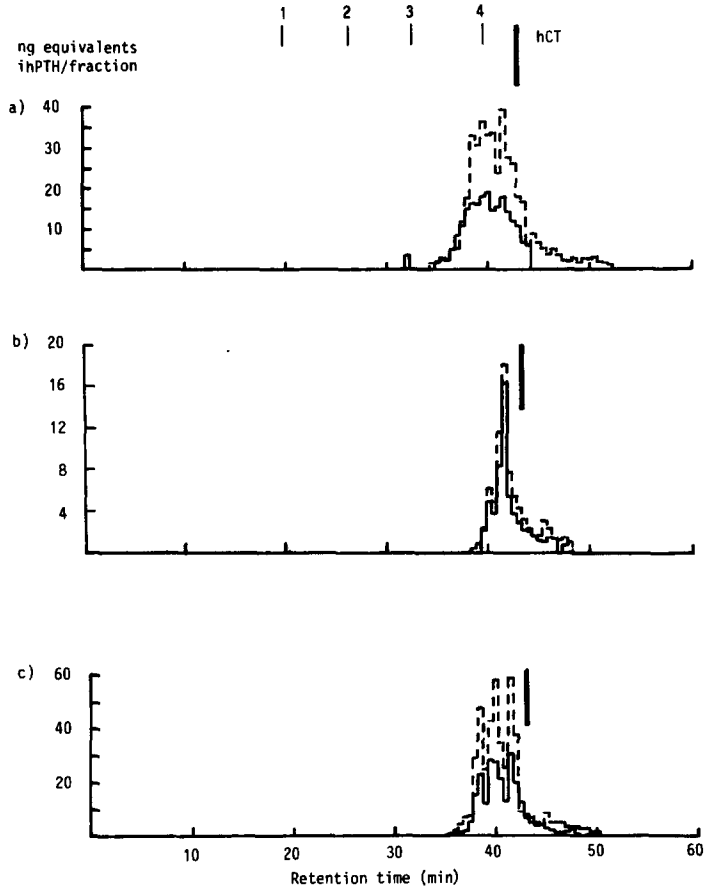

Fig. 2. Immunoreactivity profiles for the three ampouled preparations of unpurified (a, ampoule code 78/551), partially purified (b, ampoule code 75/549) and highly purified (c, ampoule code 79/500) extracts from pooled human parathyroid adenomata. RIA systems, I+N (— — —) and C+I (——) were used to assay fractions collected at 1-min intervals during gradient elution as shown in Fig. 1 and described in the text. In each instance, the contents of three ampoules (approx. 120, 75 and 300 ng respectively) were dissolved in the primary solvent for injection onto the HPLC column. The position of the hCT internal marker peptide is indicated for reference.

dures from a pool of parathyroid adenomata, were chromatographed and the eluate fractions were monitored for both N- and C-region immunoreactivity. The results illustrated in Fig. 2a, b and c show that virtually all major immunoreactivities detectable by the N- and C-region immunoassay systems are associated with a 6-min elution period, immediately preceding the hCT internal marker. There is no indication of dissociation of N- and C-immunoreactivity profiles and no dissociation of N- and C-region immunoreactivity could be demonstrated even when a shallower (0.33%/min) acetonitrile gradient was used to provide greater resolution (data not shown). However, none of these ampouled materials elute as a single sharp peak of immunoreactivity, despite the fact that the immunoreactive hCT was always localized to, at most, two consecutive eluate fractions, corresponding to the UV absorbance peak width

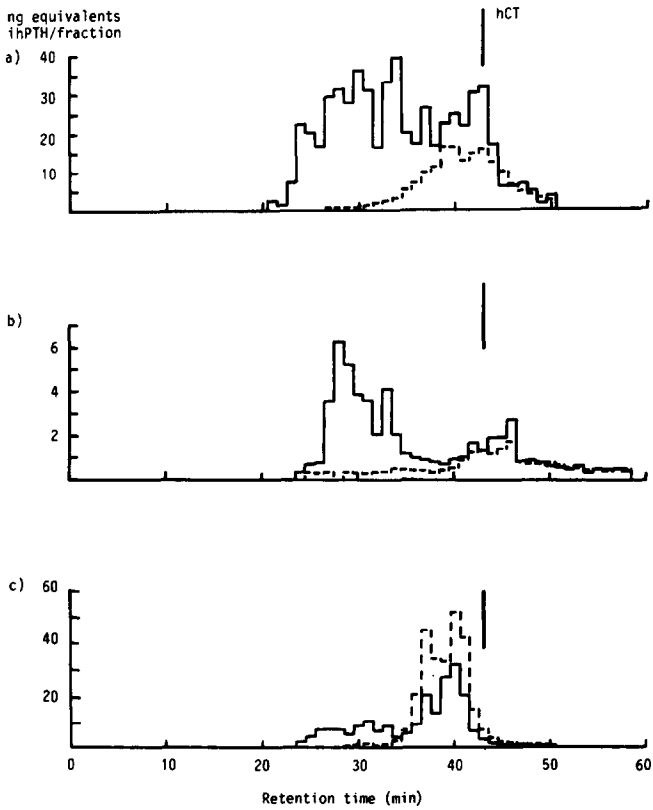

Fig. 3. Immunoreactivity profiles for three samples of biological fluids: (a) tissue culture fluid, ampoule code 78/616; (b) plasma diafiltrate, ampoule code 78/618, and (c) parathyroid cyst fluid (not ampouled). Two RIA systems, I+N (— — —) and C+I (——) were used to assay fractions collected at 1-min intervals during gradient elution shown in Fig. 1 and as described in the text. The contents of five ampoules of each of 78/616 and 78/618 were dissolved in the primary solvent for injection onto the HPLC column. The high protein content of the resulting solutions of 78/616 and 78/618 may have resulted in size exclusion phenomena not evident with other samples. Such artefacts may partly account for the apparent delay in elution of immunoreactivities in comparison with other samples. One ml of the cyst fluid had been processed through the mini-column, as described in the text, and the 20—60% acetonitrile elution step concentrated for analytical HPLC and collection of fractions. The elution position of the hCT marker is indicated.

of less than 1 min. Previous work with native bPTH, of varying degrees of purity showed that the components were readily resolved by both the chromatographic profiles and immunoreactivity profiles [5].

In contrast to these results from material prepared by conventional chemical procedures from human parathyroid tissues, there was clear evidence of the presence of hormone fragments in the biological fluids. Thus, the ampouled samples of partially-purified material from tissue culture supernatants from parathyroid adenomata (Fig. 3a), a plasma ultrafiltrate from a case of secondary hyperparathyroidism (Fig. 3b), and a sample (not ampouled) of fluid aspirated from a parathyroid cyst (Fig. 3c), gave several early-eluting peaks of C-regional immunoreactivity which were not detected with antisera with recognition of N-region immunoreactivity.

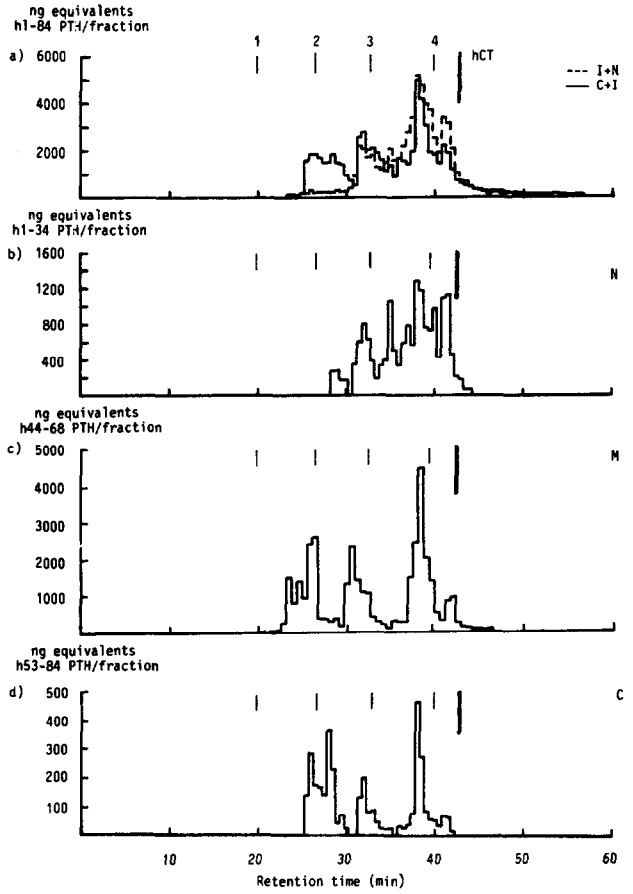

Fig. 4. I+N (– – –) and C+I (——) immunoreactivity profiles of parathyroid adenoma components on fractions collected at 0.5-min intervals (a). The gradient conditions used are shown in Fig. 1 and described in the text. Other specific region RIAs were also used to assay the same fractions, namely the homologous h1—34 PTH RIA (b), the heterologous h44—68 PTH RIA (c) and heterologous h53—84 PTH RIA (d). The elution position of the hCT marker is indicated. Small lines indicate the characteristic elution positions for the synthetic hPTH peptide fragments 44—68 (1); 53—84 (2); 28—48 (3); 1—34 (4).

It is evident from Figs. 2 and 3 that hPTH preparations processed by a variety of conventional methods show significant heterogeneity when analysed by RP-HPLC. To determine to what extent this heterogeneity was a function of type of sample and to what extent it was due to the method of purification used, a series of single human parathyroid adenomata were individually processed using only a rapid method of bulk fractionation by HPLC followed by analytical chromatography, as described in Experimental. A typical result is illustrated in Fig. 4.

Three main areas of interest can be discerned in the immunoreactivity profiles of such rapidly processed parathyroid tissue. The early-eluting materials can be seen at 25—30 min and show predominantly mid- and C-region immunoreactivity. Materials pooled from this region had no bioactivity in the canine renal membrane adenylate cyclase assay [19]. In the next period, at 30—35

min, the eluted materials are detected by N-, mid- and C-region immunoassay systems and had weak but significant bioactivity. A major area of N-, mid- and C-region immunoreactivity was evident in the later 35—40 min region, immediately preceding the hCT internal marker as in all hPTH preparations tested (cf. Figs. 2 and 3). A pooled sample from this region gave a full dose—response curve parallel to that of hPTH 1—84 in the in vitro bioassay (data not shown). Similar complex immunoreactivity profiles were obtained with separately processed tissue from two other adenomata and although the relative proportions of the different components varied (data not shown), none of these preparations gave a single, sharp peak of hPTH (N+C) immunoreactivity, when the standard bulk fractionation procedure using a mini-column, described in Experimental, was used. Other region-specific immunoassays (Fig. 4b, c and d) confirmed the heterogeneity of material prepared in this manner. Similar bioassay results were obtained on the pooled fractions from the other adenomas, confirming that the components with the longer retention times were associated with biological activity.

When, however, the evaporation of acetonitrile from the mini-column fractions was dispensed with and the samples rendered suitable for analytical chromatography with continuous gradient elution by dilution with acid saline to lower the organic modifier concentration sufficiently to allow hydrophobic interaction with the RP support, some, but not all, of the heterogeneity associated with the immunoreactivity just in front of the hCT was no longer apparent (Fig. 5). In this instance, the major immunoreactive peak eluted just 1—2 min earlier than hCT and occupied only two peak fractions corresponding to a peak width of about 1 min. This material probably, therefore, represents the intact, unmodified hPTH 1—84 and is very similar in retention time and peak width to the synthetic hPTH 1—84 (Fig. 1). The broader peaks in the hPTH from other preparations (cf. Fig. 2) must therefore be due to the presence, in this region, of other materials. We have shown previously that the oxidized forms of bPTH elute close to the unoxidized material [5] and it is a possibility, therefore, that some of the peak spreading seen in the hPTH region of some preparations is due to the presence of multiple oxidation products, as the trap fractions of 1 min were not small enough to separate all possible components as

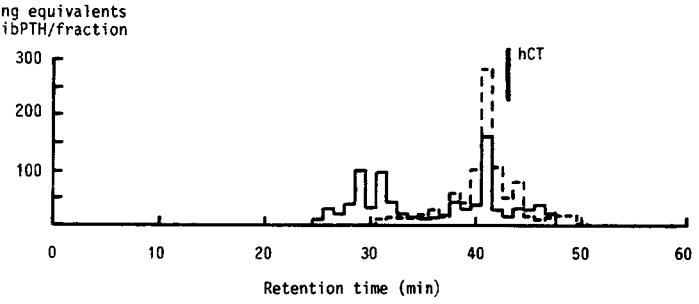

Fig. 5. I+N (— — —) and C+I (——) immunoreactivity profiles of an HPLC extraction of a single human parathyroid adenoma in which the HPLC extract was not processed (other than by dilution) prior to analytical HPLC under the gradient conditions described in the text, with collection of fractions at 1-min intervals for RIA. The position of the hCT marker is indicated.

individual peaks. It is not due to a loading effect with large amounts of biological materials as in all cases the hCT internal standard was recovered as a sharp (<1 min) peak and within two consecutive fractions by immunoassay.

DISCUSSION

To date, the characterization of products obtained from human parathyroid tissue has been restricted by the limited amounts of human tissue available and by the very low yield of peptide for chemical and biological study after the lengthy chemical extraction and purification procedures. The rapid and efficient high-yield, high-resolution RP-HPLC extraction system developed for study of endocrine tissues [2] and the HPLC fractionation systems, developed for the study of bPTH [5] have now been extended to hPTH.

Application of RP-HPLC to the analysis of the three ampouled samples of biologically active hPTH extracted from pooled human parathyroid tissue confirmed that the samples, prepared by conventional methods, precipitated with TCA and further processed by gel and ion-exchange chromatography to different degrees of purity consisted of intact immunoreactive hPTH and did not contain detectable amounts of immunoreactive cleavage products. These findings are in agreement with the results of other groups working with products extracted from human parathyroid tissue and purified by conventional methods [20—22]. The HPLC results on three ampouled preparations were thus in accord with the WHO international collaborative study which showed that homogeneity of potency estimates were obtained from a wide variety of different immunoassay systems and by in vitro bioassay systems [6].

The two ampouled samples of biological fluids, on the other hand, apparently contained little intact hormone as predicted by the marked lack of homogeneity of potency estimates obtained from immunoassay systems and low in vitro bioactivity in the WHO study. They contained multiple components with predominantly C-region immunoreactivity (Fig. 2).

However, the IRP hPTH, consisting of hPTH estimated by conventional chemical, immunochemical and biological methods to be a homogeneous product approximately 95% pure, proved to be unexpectedly heterogeneous on RP-HPLC.

Several factors might contribute to this heterogeneity of extracted intact hPTH. It could be due to the presence of a much wider spectrum of isohormones [20,21,23], and some clarification can be expected when synthesized polypeptides with alternative sequences [24,25] are available. Conformational effects do not appear to be responsible as these would affect all PTH 1—84 preparations, including the synthetic peptides.

The demonstration that rapid processing of hPTH, without evaporation, gave a sharp peak in the intact PTH region (Fig. 5) indicates that oxidation may be an important factor in generating at least some of the heterogeneity in the other preparations. The deliberate oxidation of bPTH [5] and synthetic hPTH 1—84 [26] generates a range of slightly less hydrophobic compounds.

In a previous study of hPTH by RP-HPLC, hPTH was isolated from a small pool of parathyroid adenomata [27]. Three closely associated components

were resolved. However, only one of these, the most hydrophobic, was selected for further study and cleavage and degradation products, if any, from these solid tissues were not mentioned. RP-HPLC systems with the addition of hydrophobic ion-pairing additives such as the heptafluorobutyric acid (and trifluoroacetic acid) [27] can, in some instances, resolve other components of protein mixtures [28]. Detectable heterogeneity of hPTH preparations may well, therefore, be dependent upon the mobile phase used, as well as the handling conditions and source of materials.

As noted in the Results, there appear to be three main elution areas or periods of interest when considering the immunoreactivity profiles obtained from the parathyroid tissues. The third or latest period, between 35 and 40 min, consists of immunoreactive components which are associated with heterogeneity of the intact hormone. The nature of the immunoreactive components associated with the first and second periods, i.e. eluting between 25 and 30 min and between 30 and 35 min respectively in the present studies, cannot be determined directly or indirectly. Further studies are precluded by the small amounts of human hormone available. Nevertheless, certain inferences can be drawn from the known properties of the molecule in terms of cleavage, and the predicted hydrophobic domains.

The majority of the early (25—30 min) eluting materials (see Figs. 2 and 3) could be equivalent to the larger two-thirds mid/carboxyl region fragment now generally believed to be a typical cleavage product. This interpretation is consistent with the fact that material pooled from this region showed no activation of canine renal membrane adenylate cyclase in the in vitro biological system [19].

Theoretical studies [29] indicate that the 46—55 sequence of PTH has little or no hydrophobicity and links the N- and C-terminal regions which each contain a separate hydrophobic core. Synthetic 44—68 was, in practice, the least hydrophobic of the chemically defined substances available for testing; a larger two-thirds, mid/carboxyl region of bPTH, the bovine cathepsin product, 37—84 PTH, also eluted within the 25—30 min region. These considerations would tend to support the hypothesis that the least hydrophobic components correspond to the larger mid- and C-region fragments. The possibility that parathyroid tissue may also contain or be capable of producing a variety of smaller mid- and C-region cleavage products cannot be excluded.

Human PTH products pooled from the second period, i.e. between 30 and 35 min, showed evidence of weak but significant bioactivity and were associated with N-, mid- and C-region immunoreactivities. It could be postulated that parathyroid tissue may contain or be capable of producing a variety of large N- and mid-region bioactive cleavage products although such fragments are perhaps less easy to reconcile with classical views of hPTH cleavage. There is, however, a suggestion from other workers that glandular release of fragments with mid-region but lacking C-terminal immunoreactivity may occur [30].

Definitive identification of the multiple components of hPTH which can readily be resolved by the RP-HPLC system described here, and by the system of Bennett et al. [27], is still dependent upon the availability of human parathyroid tissue, and upon micro-scale chemical analysis and biological assessment. However, it is evident that RP-HPLC systems add a major new dimension

to the future analysis of hPTH, its fragments and congeners, in tissue and biological fluids.

ACKNOWLEDGEMENT

The contribution of Mr. Duncan Purvis in assisting with many of the radioimmunoassays and in preparation of computer printouts of immunoreactivity profiles in Figs. 2—5 is gratefully acknowledged.

REFERENCES

1 M.J. O'Hare and E.C. Nice, J. Chromatogr., 171 (1979) 209.
2 E.C. Nice, M. Capp and M.J. O'Hare, J. Chromatogr., 185 (1979) 413.
3 E.C. Nice, M.W. Capp, N. Cooke and M.J. O'Hare, J. Chromatogr., 218 (1981) 569.
4 M.J. O'Hare, M.W. Capp, E.C. Nice, N.H.C. Cooke and B.G. Archer, Anal. Biochem., 126 (1982) 17.
5 J.M. Zanelli, M.J. O'Hare, E.C. Nice and P.H. Corran, J. Chromatogr., 223 (1981) 59.
6 J.M. Zanelli and R.E. Gaines-Das, J. Clin. Endocrinol. Metab., 57 (1983) in press.
7 H. Rasmussen, Y.L. Sze and R. Young, J. Biol. Chem., 239 (1964) 2852.
8 G.D. Aurbach, J. Biol. Chem., 234 (1959) 3179.
9 J.M. Zanelli and R.E. Gaines-Das, J. Endocrinol., 86 (1980) 291.
10 G. Dorn and R. Montz, Acta Endocrinol., Suppl., 208 (1977) 99.
11 W. Steibl, H. Minne, F. Raue and R. Ziegler, Horm. Metab. Res., 11 (1979) 375.
12 World Health Organisation Expert Committee on Biological Standardization, 29th Report, Annex 4, WHO Tech. Rep. Ser., 626 (1978) 108.
13 C.P.J. Caygill, Clin. Chim. Acta, 78 (1977) 507.
14 R.R. MacGregor, J.W. Hamilton, G.N. Kent, R.E. Shofstall and D.V. Cohn, J. Biol. Chem., 254 (1979) 4428.
15 T. Kimura, M. Takai, Y. Masui, T. Morikawa and S. Sakakibara, Biopolymers, 20 (1981) 1823.
16 J.M. Zanelli, B. Rafferty, B. Apostolou, G. Court and B.A.L. Hurn, J. Immunoassay, 4 (1983) in press.
17 J.M. Zanelli, B. Rafferty, R.W. Stevenson and J.A. Parsons, J. Immunoassay, 1 (1980) 289.
18 J.F. Habener and J.T. Potts, Jr., Horm. Hum. Blood: Detect. Assay 1976, 37 (1976) 589.
19 R.A. Nissenson, S.R. Abbott, A.P. Teitelbaum, O.H. Clark and C.D. Arnaud, J. Clin. Endocrinol. Metab., 52 (1981) 840.
20 H.T. Keutmann, P.M. Barling, G.N. Hendy, G.V. Segre, H.D. Niall, G.D. Aurbach, J.T. Potts, Jr. and J.L.H. O'Riordan, Biochemistry, 13 (1974) 1646.
21 H.T. Keutmann, G.N. Hendy, M. Boehnert, J.L.H. O'Riordan and J.T. Potts, Jr., J. Endocrinol., 78 (1978) 49.
22 F.P. Di Bella, J.B. Gilkinson, J. Fleuck and C.D. Arnaud, J. Clin. Endocrinol. Metab., 46 (1978) 604.
23 H.T. Keutmann, G.D. Aurbach, B.F. Dawson, H.D. Niall, L.J. Deftos and J.T. Potts, Jr., Biochemistry, 10 (1971) 2779.
24 H.T. Keutmann, M.M. Sauer, G.N. Hendy, J.L.H. O'Riordan and J.T. Potts, Jr., Biochemistry, 17 (1978) 5723.
25 G.N. Hendy, H.M. Kronenberg, J.T. Potts, Jr. and A. Rich, Proc. Nat. Acad. Sci. U.S., 78 (1981) 7365.
26 S. Sakakibara, personal communication.
27 H.P.J. Bennett, S. Solomon and D. Goltzman, J. Biochem., 197 (1981) 391.
28 M.J. O'Hare, J. Smith, B.G. Archer, N.H.C. Cooke and E.C. Nice, Protides Biol. Fluids, 30 (1983) 723.
29 J.E. Zull and N.B. Lev, Proc. Nat. Acad. Sci. U.S., 77 (1980) 3791.
30 S.J. Marx, M.F. Sharp, A. Krudy, M. Rosenblatt and L.E. Mallette, J. Clin. Endocrinol. Metab., 53 (1981) 76.