Isolation and analysis of human parathyrin in parathyroid tissue and plasma

Use of reversed-phase liquid chromatography

Hugh P. J. BENNETT, Samuel SOLOMON and David GOLTZMAN*

Department of Medicine, McGill University, Montreal, and Department of Medicine, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1

(Received 16 February 1981/Accepted 26 February 1981)

Reversed-phase liquid chromatography techniques have been used to extract and purify human parathyrin from parathyroid adenomas and to analyse the circulating forms of human parathyrin in plasma. Both the supernatant from tissue homogenates, and plasma were extracted with octadecylsilyl-silica (ODS-silica) in a batch procedure. Extracts were subjected to reversed-phase high-pressure liquid chromatography (h.p.l.c.) employing solvent systems composed of aqueous acetonitrile containing trifluoroacetic acid or heptafluorobutyric acid as hydrophobic ion-pairing reagents. The volatile solvents facilitated the radioimmunoassay, bioassay *in vitro* and amino acid analysis of column fractions and permitted monitoring for u.v. absorbance at 210 nm. Isolated glandular parathyrin was found to be homogeneous by sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis, to have an amino acid composition conforming to that of human parathyrin-(1–84)-tetraoctacontapeptide and to be bioactive in both renal adenylate cyclase and cytochemical bioassays. ODS-silica extraction permitted examination of large plasma samples by reversed-phase h.p.l.c., facilitating the resolution of the various circulating molecular forms of parathyrin according to their hydrophobic character. Because of its rapidity, excellent recovery and high resolving power, the methodology utilized is uniquely suited to the purification and analysis of parathyrin in tissues and body fluids.

The availability of purified human parathyrin for biological studies has been restricted by the low concentrations of the hormone in parathyroid tissue and the small size and limited quantities of glands available for extraction (Keutmann *et al.*, 1974, 1978b). Furthermore, analyses of parathyrin in body fluids has been complicated by low concentrations and by multiple molecular forms (Berson & Yalow, 1968; Segre *et al.*, 1972; Canterbury & Reiss, 1972; Goldsmith *et al.*, 1973; Silverman & Yalow, 1973; Goltzman *et al.*, 1980). To overcome these obstacles, initial extraction with ODS-silica followed by reversed-phase h.p.l.c. has been employed to achieve high recovery and enhanced resolution in the rapid purification of parathyrin from parathyroid

adenomas and in the analysis of the circulating hormone. The results document the utility of this technique in isolating parathyrin from small amounts of tissues and in fractionating circulating hormone for subsequent analysis.

Experimental

Hormone preparations

Highly purified native bovine parathyrin-(1–84)-tetraoctacontapeptide used as standard in the bioassays for potency determinations was M.R.C. Research Standard 72/286, National Institute for Biological Standards and Control (N.I.B.S.C.). By bioassays *in vivo* (Parsons *et al.*, 1973) ampoules of this preparation contained 520 i.u. in terms of the International Reference Preparation for (bovine) parathyrin for bioassay. An approximate estimate of the peptide content of each ampoule was 200 µg bovine parathyrin-(1–84)-peptide. Native human parathyrin-(1–84)-peptide used as a radioimmunoassay standard was N.I.B.S.C. code 75/549. Each ampoule contained the equivalent of 25 ng of very

Abbreviations used: ODS, octadecylsilyl; h.p.l.c., high-pressure liquid chromatography; SDS, sodium dodecyl sulphate.

* To whom reprint requests should be addressed at: Department of Medicine, Room M9.28, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Que., Canada H3A 1A1.

highly purified human parathyrin (Zanelli & Gaines-Das, 1980). For all other studies, purified (bovine) parathyrin-(1-84)-peptide (approx. 2500 M.R.C. units/mg) was employed, which was kindly supplied by Dr. H. T. Keutmann and Dr. J. T. Potts, Jr., Endocrine Unit, Massachusetts General Hospital, Boston, MA, U.S.A. Synthetic human parathyrin-(1-34)-peptide and human parathyrin-(53-84)-peptide were gifts of Dr. M. Rosenblatt and Dr. J. T. Potts, Jr., and bovine parathyrin-(1-34)-peptide (approx. 6000 units/mg) was purchased from Beckman Instruments, Spinco Division, Palo Alto, CA, U.S.A.

Extraction of parathyroid tissue

Parathyroid adenomas used in the study were collected over a 2-year period (1978-1979). Tissue was obtained as soon as possible after surgical removal and stored frozen at -70°C . Extraction of parathyrin from glandular tissue was by a modification of a previously described method (Bennett *et al.*, 1977, 1980b,c) involving repeated homogenization at 0°C in a medium consisting of 1M-HCl containing 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) trifluoroacetic acid. The homogenate supernatants were then extracted using cartridges of ODS-silica as described below.

In initial experiments, single adenomas (0.4 g wet wt. of tissue) were homogenized at -10°C sequentially in acetone (20 ml/g), n-hexane (10 ml/g) and finally acetone (20 ml/g) (Keutmann *et al.*, 1974). Residual acetone was removed from the dehydrated and defatted tissue by a light water vacuum. This dried material was then extracted with homogenization medium and the combined homogenate supernatants were passed through a single ODS-silica cartridge. This extraction procedure for single adenomas was also the basis of the preparative isolation of human parathyrin from multiple adenomas, which was modified by augmented extraction with homogenization medium (7×40 ml) and with the use of seven ODS-silica cartridges.

Plasma preparation

Peripheral venous blood was obtained from the antecubital vein from patients with either primary hyperparathyroidism (resulting from benign adenoma or carcinoma) and normal renal function, or from a patient with secondary hyperparathyroidism and chronic renal failure (on chronic haemodialysis). The diagnosis of parathyroid carcinoma was made clinically at the time of surgery when invasion of the trachea by parathyroid tissue was observed, was confirmed histologically by examination of resected tissue and the malignant nature of the tumour was again indicated by evidence of recurrence post-operatively. Blood was

also obtained from the parathyroid venous effluent of a patient with severe secondary hyperparathyroidism, complicating x-linked hypophosphataemic rickets, who had normal renal function and who had undergone a total parathyroidectomy and left forearm parathyroid transplant (Wells *et al.*, 1976). Blood was obtained for chromatography from the left antecubital vein 3 months after transplantation when a 12-fold increase in the immunoreactive concentration of parathyrin was demonstrable in samples obtained from the left antecubital vein relative to samples obtained from the right. Venous blood was drawn into heparinized syringes, centrifuged (500 g for 20 min) and stored frozen at -20°C until extracted (1-3 weeks). Plasma was passed through ODS-silica cartridges at 4°C in batches of 3 ml to prevent overloading with protein.

Extraction of tissue homogenate supernatants and plasma with ODS-silica

ODS-silica was used to extract peptides from both tissue homogenate supernatants and plasma in a batch procedure similar to the method previously described (Bennett *et al.*, 1977, 1978, 1980c). This was performed using Waters Associates Sep-Pak C₁₈ cartridges (Waters Scientific, Mississauga, Ont., Canada). The ODS-silica was first 'wetted' with organic solvent to permit the aqueous phase to interact efficiently with the hydrophobic surface, by priming the cartridges with 5 ml of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Excess solvent was removed by passing 5 ml of 0.1% trifluoroacetic acid through each cartridge before applying the tissue extract or plasma. After loading the biological sample, 20 ml of 0.1% trifluoroacetic acid was passed through to remove residual protein and homogenization medium. Elution of the ODS-silica cartridges with 3 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid produced a preparation suitable for reversed-phase h.p.l.c. as described below.

Apparatus for h.p.l.c. A Waters Associates h.p.l.c. system was used in this study, which consisted of two 6000A pumps controlled by a solvent programmer. Chromatography was carried out using C₁₈ μ Bondapak h.p.l.c. columns. The h.p.l.c. apparatus and columns were purchased from Waters Scientific, Mississauga, Ont., Canada. Column eluates were monitored for u.v. absorbance using variable-wavelength flow-through spectrophotometers (Perkin-Elmer, Montreal, Que., Canada), connected in series. The model LC-55 (0.6 cm pathlength cell) instrument measured absorbance at 278 nm and the model LC-75 (1 cm pathlength cell) instrument at 210 nm. Column fractions were collected every minute in borosilicate disposable glass test tubes (12 mm $\times$ 75 mm) using an LKB Ultrarac 7000

fraction collector (Fisher Scientific, Montreal, Que., Canada) and were stored at -20°C .

Preparation of h.p.l.c. solvents. Trifluoroacetic acid (laboratory reagent grade) was obtained from BDH Chemicals, Poole, Dorset, U.K. Heptafluorobutyric acid (purissimum grade) was purchased from Tridom Chemical Inc., Hauppauge, NY, U.S.A., and acetonitrile (h.p.l.c. grade) was from Fisher Scientific, Waltham, MA, U.S.A. Water and solvents for h.p.l.c. were prepared as described previously (Bennett *et al.*, 1980a).

Solvent A, consisting of 0.1% trifluoroacetic acid or 0.13% heptafluorobutyric acid, and solvent B, consisting of 80% aqueous acetonitrile, containing 0.1% trifluoroacetic acid or 0.13% heptafluorobutyric acid, were delivered by the two h.p.l.c. pumps, and linear gradients of increasing acetonitrile concentration were formed under the control of the solvent programmer. Operating pressure was generally in the range 1000–3000 lb/in² (6.9–20.7 MPa) and varied with the type of column used, the solvent concentration and the flow rate.

Loading of h.p.l.c. column. ODS-silica cartridge eluates and column fractions were diluted with either 0.1% trifluoroacetic acid or 0.13% heptafluorobutyric acid (depending on the h.p.l.c. solvent system used in the subsequent step) and pumped directly on to the h.p.l.c. column using one of the unused ports on the 'aqueous' pump (i.e. the h.p.l.c. pump normally delivering 0.1% trifluoroacetic acid or 0.13% heptafluorobutyric acid) at a flow rate of 3 ml/min. Before loading in this manner the column was equilibrated with 12% acetonitrile (containing 0.1% trifluoroacetic acid or 0.13% heptafluorobutyric acid where appropriate), and after loading was again equilibrated with 10 ml of this solution to wash the sample through the h.p.l.c. system on to the column. Before commencing gradient elution the 'aqueous' pump was flushed with 0.1% trifluoroacetic acid or 0.13% heptafluorobutyric acid.

Reversed-phase h.p.l.c. of tissue extracts. The preliminary single adenoma extracts (ODS-silica eluates of 3 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid) were diluted 1:4 with 0.1% trifluoroacetic acid and pumped on to the C₁₈ μ Bondapak column as described above. The column was developed with a linear gradient of 20–50% acetonitrile containing 0.1% trifluoroacetic acid throughout over 90 min at a flow rate of 1.5 ml/min. Fractions containing the main peak of immunoreactive parathyrin were diluted 1:1 with 0.1% trifluoroacetic acid and pumped on to the C₁₈ μ Bondapak column. The column was eluted isocratically with 32% acetonitrile containing 0.1% trifluoroacetic acid and a single somewhat broad major peak of immunoreactivity was observed that was of less than optimal bioactivity. Consequently the purification of human parathyrin from a large-

scale tissue extraction (pool of 12 parathyroid adenomas) was a three-step procedure. In the first step, the ODS-silica eluates (21 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid) were diluted 1:4 with 0.1% trifluoroacetic acid and pumped on to the C₁₈ μ Bondapak column. The column was eluted with a linear gradient of 20–50% acetonitrile containing 0.1% trifluoroacetic acid throughout over 90 min at a flow rate of 1.5 ml/min. Fractions containing the main peak of parathyrin immunoreactivity were diluted 1:2 with 0.13% heptafluorobutyric acid and, in the second step, pumped on to the C₁₈ μ Bondapak column. The column was eluted with a linear gradient of 24–56% acetonitrile containing 0.13% heptafluorobutyric acid throughout over 60 min at a flow rate of 1.5 ml/min. Fractions containing the main peak of parathyrin immunoreactivity were diluted 1:2 with 0.1% trifluoroacetic acid and pumped on to the C₁₈ μ Bondapak column. In this third and final step the column was eluted with a linear gradient of 28–36% acetonitrile containing 0.1% trifluoroacetic acid over 60 min at a flow rate of 1.5 ml/min.

Reversed-phase h.p.l.c. of plasma extracts

ODS-silica eluates (80% acetonitrile containing 0.1% trifluoroacetic acid) were diluted 1:4 with 0.13% heptafluorobutyric acid and pumped on to the C₁₈ μ Bondapak column. The column was eluted in each case with a linear gradient of 28–48% acetonitrile containing 0.13% heptafluorobutyric acid over 60 min at a flow rate of 1.5 ml/min.

Preparation of column eluates for analysis

Portions of column fractions were evaporated in the liquid state using a National Vacuum oven (Fisher Scientific) at 37°C using a water vacuum. By chilling the samples to -10°C for 30 min, a vacuum could be applied without bumping of even whole fractions (i.e. 1.5 ml). Such dried portions were then employed for bioassay, radioimmunoassay or amino acid analysis.

Radioimmunoassays

Radioimmunoassays for parathyrin were carried out as previously described (Goltzman *et al.*, 1976b) employing ¹²⁵I-labelled bovine parathyrin-(1–84)-peptide as tracer and dextran-charcoal for phase separation. Dried portions of column fractions were taken up in plasma known to have no detectable parathyrin and assayed undiluted or in appropriate dilution in duplicate. For tissue extraction studies, human parathyrin-(1–84)-peptide was used as assay standard. Three different antisera from Burroughs-Wellcome, London, U.K., were employed. With antiserum AS 211/41, human parathyrin-(1–34)-peptide, and human parathyrin-(53–84)-peptide, each inhibited the binding of ¹²⁵I-labelled bovine

parathyrin-(1-84)-peptide by approx. 50%. Therefore this was considered to be a multivalent antiserum. With antiserum AS 211/32, human parathyrin-(1-34)-peptide inhibited the binding of tracer by over 75%, whereas only minimal inhibition was seen with human parathyrin-(53-84)-peptide; this therefore recognized antigenic determinants mainly in the *N*-terminal region of parathyrin-(1-84)-peptide and was considered to be an *N*-terminal antiserum. With antiserum AS 1127/23, human parathyrin-(53-84)-peptide inhibited tracer binding by over 70%, whereas minimal inhibition was seen with human parathyrin-(1-34)-peptide; this therefore recognized antigenic determinants in the *C*-terminal-region of parathyrin-(1-84)-peptide, and was considered to be a *C*-terminal antiserum.

Bioassays

Adenylate cyclase activity was assayed as previously described (Goltzman *et al.*, 1975b) by measuring the conversion of [α - 32 P]ATP into cyclic [32 P]AMP at 37°C after 10 min incubation with rabbit renal cortical membranes. Purified membranes from the kidneys of male albino (New Zealand) rabbits were prepared by a method previously described (Goltzman *et al.*, 1976b).

Activity in a cytochemical bioassay for parathyrin was determined as previously described (Chambers *et al.*, 1978; Goltzman *et al.*, 1980). Renal cortical segments from Hartley-strain albino guinea pigs (450-550 g) were first incubated for 5 h at 37°C in non-proliferative synthetic culture medium (Trowell's T8 medium; Gibco, Grand Island, NY, U.S.A.) in an atmosphere of O₂/CO₂ (19:1). After incubation with fresh medium for 8 min, segments were further cultured for 6 min at 37°C with graded doses of either hormone standard or unknown in fresh Trowell's T8 medium containing 1% (v/v) plasma and then immediately chilled by immersion in *n*-hexane at -70°C. Segments were then cut into 16 μ m sections and unfixed sections were tested for glucose 6-phosphate dehydrogenase activity employing a reaction medium containing 5 mM-glucose 6-phosphate, 3 mM-NADP⁺, 0.67 mM-phenazine methosulphate, 5 mM-neotetrazolium chloride, 10 mM-KCN and poly(vinyl alcohol) (12%) in 0.05 M-glycylglycine buffer, pH 8.0, for 5 min. The reduced neotetrazolium produced, an intensely coloured formazan, was then measured in individual convoluted tubule cells with a Vickers M85 scanning and integrating microdensitometer at 585 nm.

Polyacrylamide-gel electrophoresis

Portions of extracted and purified human hormone were analysed by electrophoresis in SDS/urea/acrylamide gels that contained 10% acrylamide, 1.5% bisacrylamide, 0.1% SDS, 8 M-urea and 0.1 M-sodium phosphate buffer, pH 7.2 (Goltzman *et*

al., 1976a). Standards employed were native bovine parathyrin-(1-84)-peptide (mol.wt. 9562), and myoglobin and CNBr-cleaved fragments of myoglobin of mol.wts. 2512, 6214, 8159, 14 404 and 16 949, which were purchased from BDH Chemicals.

Amino acid analysis

Approx. 10 μ g of isolated human parathyrin was hydrolysed in an evacuated glass tube in 0.25 ml of 6 M-HCl (with a crystal of phenol to prevent tyrosine loss) for 18 h at 110°C. Amino acid analysis was performed on a Durrum Series D500 analyser.

Results

Tissue extractions

Extraction and purification of parathyrin from multiple adenomas. The use of 12 parathyroid adenomas (total wet wt. 11.1 g) resulted in a defatted and dehydrated preparation weighing 2 g. Employing a multivalent radioimmunoassay to bovine parathyrin-(1-84)-peptide, 480 μ g-equiv. of parathyrin was extracted from dried and defatted material into the homogenization medium; over 90% was recovered with the first homogenization and less than 10% of this quantity was lost during the initial drying and defatting steps. Approx. 85% of hormone added to the ODS-silica cartridges were specifically eluted with 80% acetonitrile containing 0.1% trifluoroacetic acid. Purification of the human parathyrin was a three step procedure using the same h.p.l.c. column (C₁₈ μ Bondapak) at each stage but employing first 0.1% trifluoroacetic acid as counter-ion (Fig. 1a), and then 0.13% heptafluorobutyric acid as counter-ion (Fig. 1b). Finally the major immunoreactive component was rerun using a shallow gradient of acetonitrile with 0.1% trifluoroacetic acid again used as counter-ion (Fig. 1c). Immunoreactive parathyrin now coincided exactly with a u.v. absorbing component and was considered to be homogeneous.

Assessment of purity and in vitro bioactivity of extracted parathyrin. The immunoreactive peak was found to migrate as a single homogenous band on polyacrylamide/SDS/urea-gel electrophoresis, to co-migrate with purified native bovine parathyrin-(1-84)-peptide in this system and to have an apparent molecular weight, relative to defined standards, appropriate to human parathyrin-(1-84)-peptide (Fig. 2).

The amino acid composition of the purified peak after acid hydrolysis conformed to the published composition of human parathyrin-(1-84)-peptide, isolated and purified by gel filtration and ion-exchange chromatography (Keutmann *et al.*, 1974, 1978a,b).

Fig. 1. Reversed-phase h.p.l.c. of an ODS-silica extract of 12 pooled human parathyroid adenomas

The column (C_{18} μ Bondapak) was first eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (—) as described in the Experimental section (a). Pooled fractions 30–34 containing the main peak of immunoreactive parathyrin shown in (a) were then re-chromatographed on the column (C_{18} μ Bondapak) eluting with a linear gradient of acetonitrile containing 0.13% heptafluorobutyric acid (—) (b). Pooled fractions 43–45 containing the main peak of immunoreactive parathyrin shown in (b) were then re-chromatographed on the column eluting with a shallower linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (—) (c). The column eluate at each phase was monitored for u.v. absorbance at 210nm (---) and 280nm (....). Parathyrin immunoreactivity (iPTH) was measured in each eluted fraction using a multivalent antiserum and is expressed as μ g-equiv./min (—).

Bioassay of the purified hormone was performed in two systems (Fig. 3). In a renal adenylate cyclase assay *in vitro*, the potency of the preparation relative to bovine parathyrin-(1–84)-peptide, M.R.C. code no. 72/286, was 295 (285–310) units/mg (mean potency with 95% confidence-limit values), whereas in a renal cytochemical bioassay *in vitro*, relative to the same standard, the potency was 4175 (2500–5850) units/mg (mean potency with 95% confidence-limit values). These potency deter-

minations were based on the mass of peptide determined by amino acid analysis (Fig. 3).

Plasma analyses

Recovery studies. In initial studies 25ng of purified human parathyrin-(1–84)-peptide from this study and 25ng of synthetic human parathyrin-(1–34)-peptide were each added to 3ml of hypoparathyroid plasma and extracted separately with cartridges of ODS-silica. Radioimmunoassay, em-

Fig. 2. Polyacrylamide-gel electrophoresis of human parathyrin purified by reversed-phase h.p.l.c.

A single homogeneous band was seen on electrophoresis of this material in a 10% polyacrylamide disc gel containing 2% SDS/8M-urea stained with Coomassie Blue (a, gel B). This band co-migrated with the major band of the preparation of bovine parathyrin-(1-84)-peptide used as a standard (a, gel C). Myoglobin and CNBr-cleaved myoglobin fragments of mol.wts. 16949, 14404, 8159, 6214 and 2512 were also employed as molecular-weight standards (a, gel A; bands running from top to bottom respectively). The purified human parathyrin (b, arrow) was estimated with these six standards (O) to have the same molecular weight as bovine parathyrin-(1-84)-peptide (b). Electrophoresis was performed as described in the Experimental section.

Fig. 3. Bioactivity of human parathyrin-(1-84)-peptide purified by reversed-phase h.p.l.c. in renal adenylate cyclase (a) and renal cytochemical (b) bioassays

Effects of increasing concentrations of human parathyrin-(1-84)-peptide (O) were compared with effects of increasing concentrations of bovine parathyrin-(1-84)-peptide research standard (M.R.C. 72/286; 2500 units/mg; □) on adenylate cyclase activity in rabbit renal cortical membranes (a) and on glucose 6-phosphate dehydrogenase (G6PD) activity in guinea-pig renal cortical segments (b). Adenylate cyclase and cytochemical bioassays were performed as described in the Experimental section. Each point in (a) represents the mean $\pm$ S.E.M. for triplicate determinations and each point in (b) represents the mean $\pm$ S.E.M. for 20 measurements from duplicate sections. B indicates the basal activity of each assay. Abbreviation used: PTH, parathyrin.

Table 1. Extraction of exogenous parathyrin added to 3 ml of hypoparathyroid plasma

Details of the extraction procedure employing cartridges of ODS-silica are described in the text. Quantities of immunoreactive hormone initially detected in hypoparathyroid plasma with a multivalent antiserum were taken as 100%.

	Human parathyrin-(1-84)-peptide		Human parathyrin-(1-34)-peptide	
	(ng)	(%)	(ng)	(%)
Plasma content	24.00	100	23.00	100
ODS cartridge flow-through	0.90	—	0.96	—
ODS cartridge elution	23.00	96	21.5	93

Fig. 4. Reversed-phase h.p.l.c. of native human parathyrin-(1-84)-peptide and synthetic human parathyrin-(1-34)-peptide

Each peptide had been added to 3 ml portions of hypoparathyroid plasma and extracted separately with ODS-silica. The ODS-silica eluates were combined and applied to the column (C₁₈ μ Bondapak), which was eluted with a linear acetonitrile gradient containing 0.13% heptafluorobutyric acid (---) as described in the Experimental section. Parathyrin immunoreactivity (iPTH) was determined using a multivalent antiserum and is expressed as ng-equiv./min (—). D indicates the detection limit of the radioimmunoassay. The solid arrow indicates the elution position of human parathyrin-(1-84)-peptide and the broken arrow the elution position of human parathyrin-(1-34)-peptide.

greater than 90% in each case (Table 1). Studies of the extraction and recovery of endogenous hormone in hyperparathyroid plasma yielded similar results. Purified human parathyrin-(1-84)-peptide and synthetic human parathyrin-(1-34)-peptide eluted from ODS-silica cartridges were then combined and subjected to reversed-phase h.p.l.c. as described in the Experimental section. Two discrete peaks were obtained, one somewhat broader peak corresponding to the elution position of human parathyrin-(1-34)-peptide and one corresponding to that of human parathyrin-(1-84)-peptide (Fig. 4). Total immunoreactivity recovered was 88% for human parathyrin-(1-34)-peptide and 90% for human parathyrin-(1-84)-peptide. The breadth of the human parathyrin-(1-34)-peptide peak may have resulted from co-elution of oxidized forms of the peptides generated during storage.

Analysis of plasma from hyperparathyroid patients. Plasma was analysed by reversed-phase h.p.l.c. as described in the Experimental section. Immunoassays employing appropriate antisera were used to determine N- and C-terminal immunoreactive parathyrin in each column fraction. In each hyperparathyroid plasma a major peak of N-terminal immunoreactivity was observed coinciding with a peak of C-terminal immunoreactivity and eluting in the position of human parathyrin-(1-84)-peptide (Fig. 5). In each case only a very small peak or shoulder of N-terminal immunoreactivity with no associated C-terminal immunoreactivity eluted in the position of human parathyrin-(1-34)-peptide (Fig. 5). Multiple peaks of C-terminal immunoreactivity were seen in each case, without any corresponding N-terminal immunoreactivity. Least C-terminal immunoreactivity was detected in parathyroid venous effluent plasma (Fig. 5b) and most in the peripheral plasma of the patients with uraemia (Fig. 5c) and a parathyroid carcinoma (Fig. 5d) respectively. C-terminal immunoreactivity eluting after human parathyrin-(1-84)-peptide was seen in substantial quantities only in plasma from the patient with the parathyroid carcinoma (Fig. 5d) and only to a minimal extent in parathyroid venous effluent plasma (Fig. 5b).

employing a multivalent antiserum, was used to monitor hormonal recovery at each step. Most of the native and synthetic hormones added in plasma were retained by the ODS-silica and the recovery of immunoreactivity after elution was found to be

Fig. 5. Reversed-phase h.p.l.c. of ODS-silica extracts of hyperparathyroid plasma

The column (C_{18} μ Bondapak) was eluted in each case with a linear gradient of acetonitrile containing 0.13% heptafluorobutyric acid (—) as described in the Experimental section. Parathyrin immunoreactivity (iPTH) was determined separately with *N*-terminal-directed (—) and *C*-terminal-directed (---) antisera in each column fraction and expressed as pg-equiv./min. Total *N*-terminal and total *C*-terminal immunoreactivity represent that recovered from 1.5 ml of chromatographed plasma. D indicates the detection limits of the radioimmunoassays. Hormonal forms were analysed from peripheral plasma of a patient with primary hyperparathyroidism and normal renal function (a); from the parathyroid venous effluent of a patient with hyperparathyroidism and x-linked hypophosphataemic rickets, who had undergone a total parathyroidectomy and forearm parathyroid transplant (b), from peripheral plasma of a patient with chronic renal failure, on haemodialysis, with secondary hyperparathyroidism (c) and from peripheral plasma of a patient with a parathyroid carcinoma and normal renal function (d). The solid arrow indicates the elution position of human parathyrin-(1-84)-peptide, and the broken arrow the elution position of human parathyrin-(1-34)-peptide. Details of the clinical conditions and plasma preparation are provided in the Experimental section.

Discussion

The use of ODS-silica for batch extraction of parathyrin from supernatants of homogenates of adenomas was facilitated by the high affinity of ODS-silica for this peptide in aqueous solution and by the high recovery of the hormone achieved by elution with 80% acetonitrile containing 0.1% trifluoroacetic acid. In view of the low capacity of the ODS-silica for high-molecular-weight protein (Lewis *et al.*, 1980) and its low affinity for salts and other polar materials, an extract was thereby produced, which was desalted and largely deproteinized. Loading of the reversed-phase h.p.l.c. column was then readily achieved by diluting the extract such that the acetonitrile concentration was below the starting concentration of this component of the mobile phase. Inasmuch as large loading

volumes could be tolerated by the column at this solvent concentration with little effect of retention times, the need for decreasing sample size before injection with its attendant losses could be obviated. Consequently 85% of immunoreactive material extracted from dried and defatted glands could be utilized for chromatographic purification. In this respect, the methodology described appears to have a substantial advantage over standard techniques employing trichloroacetic acid before column purification, where relatively heavy losses have been experienced (Keutmann *et al.*, 1978a).

For all chromatographic steps columns were eluted with linear gradients of acetonitrile containing trifluoroacetic acid or heptafluorobutyric acid as counter-ions. At the concentrations used, each acid solution was sufficiently u.v. transparent to facilitate monitoring of column eluates at 210 nm.

Furthermore the use of volatile buffer systems permitted direct radioimmunoassay, bioassay and amino acid analysis of column fractions after drying *in vacuo* without the need for desalting with its associated losses.

For the extraction of multiple adenomas, initial reversed-phase h.p.l.c. using trifluoroacetic acid as a counter-ion (Fig. 1a) was followed by chromatography using heptafluorobutyric acid as a more hydrophobic counter-ion (Fig. 1b). Heptafluorobutyric acid has been previously shown to alter retention times relative to trifluoroacetic acid depending on the number of positive charges within each peptide available for ion-pairing, retention time increasing with increasing numbers of basic charges (Bennett *et al.*, 1980a). The essentials of ion-exchange chromatography were thus introduced into the procedure. In the final chromatographic step a shallower acetonitrile gradient was employed, reverting to trifluoroacetic acid as a counter-ion (Fig. 1c).

This strategy yielded a final purified product, which was found to be homogeneous by polyacrylamide/SDS/urea-gel electrophoresis with a molecular weight corresponding to the known molecular weight of the major glandular form of parathyrin (Fig. 2). Amino acid composition confirmed the identity of the material as human parathyrin-(1-84)-peptide and bioactivity of the purified hormone in two renal bioassay systems *in vitro* (Fig. 3) was comparable with that which has been reported previously for human parathyrin-(1-84)-peptide extracted and purified by conventional techniques (Keutmann *et al.*, 1974; Chambers *et al.*, 1978). The 14-fold difference in potency *in vitro* obtained with respect to the same reference standard in the two renal bioassays appears to reflect the marked sensitivity of the adenylate cyclase assay *in vitro* to alterations at the *N*-terminus. Thus the presence of alanine at the *N*-terminus of the bovine molecule in place of serine at the *N*-terminus of the human hormone appears to confer a marked increase in potency of the bovine over the human hormone in the adenylate cyclase assay *in vitro* (Keutmann *et al.*, 1974; Goltzman *et al.*, 1975a). This sensitivity to the *N*-terminal residue does not appear to be reflected *in vivo* (Parsons *et al.*, 1975), or in the cytochemical assay *in vitro* (Chambers *et al.*, 1978).

One major advantage of the present techniques described for obtaining parathyrin would therefore be the decrease of hormonal losses during extraction and purification. Recovery of total immunoassayable hormone from the column purifications was consistently found to be greater than 80%, compared with standard techniques of gel filtration and ion-exchange chromatography with reported recoveries of 60% and 50% respectively (Keutmann *et al.*, 1974, 1978a). Additionally, overall yields of

highly purified intact hormone obtained by the present techniques (approx. 10% of total immuno-reactivity extracted from the dried and defatted glands) seem to compare very favourably with reported methods of obtaining similar human hormone (Keutmann *et al.*, 1974). A second major advantage would include the rapidity with which extraction and purification can be achieved relative to existing methods, the rate-limiting factor in the present studies having been the delay ensuing in monitoring the procedures by radioimmunoassay. These considerations would seem to be of particular importance in extraction, analysis and purification of human parathyrin, which is prevalent in tissues in relatively small quantities.

Considerable interest has centred on the molecular circulating forms of parathyrin (Berson & Yalow, 1968; Segre *et al.*, 1972; Canterbury & Reiss, 1972; Goldsmith *et al.*, 1973; Silverman & Yalow, 1973; Goltzman *et al.*, 1980). This interest has evolved from the discovery, from structure-function studies of parathyrin, that the entire bioactivity of the molecule seems to reside within the *N*-terminal region (Tregear *et al.*, 1973; Goltzman *et al.*, 1978) and from a variety of studies that indicate that the major circulating moiety of parathyrin is not the major glandular form, parathyrin-(1-84)-peptide, but is a middle and *C*-terminal, and presumably inactive, fragment of the hormone (Segre *et al.*, 1972; Canterbury & Reiss, 1972; Goldsmith *et al.*, 1973; Silverman & Yalow, 1973). The present studies assessed the utility of fractionation of plasma by reversed-phase liquid-chromatography techniques for further elucidation of the nature of the circulating parathyrin moieties.

The studies revealed that the major circulating hormonal form detectable by *N*-terminal-directed radioimmunoassay (and therefore the major presumed bioactive form detected) in all pathological states examined was similar to, or identical with, the major glandular form of the hormone. This was apparent in that in each case the *N*-terminal immunoreactive form also contained sufficient *C*-terminal antigenic determinants to be recognized by *C*-terminal-directed radioimmunoassay and in that it eluted in the position of native parathyrin-(1-84)-peptide. This then supports previous work directly bioassaying column effluents of plasma fractionated by gel filtration (Goltzman *et al.*, 1980).

In all column profiles, total *C*-terminal immuno-reactivity exceeded total *N*-terminal immuno-reactivity, although this was most apparent in the plasma obtained from patients with chronic renal failure and parathyroid carcinoma respectively. The identity of these *C*-terminal moieties and the issue of the existence of corresponding *N*-terminal fragments require further study. However, subsequent analyses should be aided by the capacity to employ

ODS-silica for extraction of relatively large volumes of plasma and by the enhanced resolution and recovery of the different hormonal forms that can be achieved with the reversed-phase liquid chromatography system employed.

The excellent technical assistance of Miren Gratton, Linda Melnbardis and Janice Lord is gratefully acknowledged as is the excellent secretarial assistance of Ruth Sembera. We also thank Dr. M. Wexler and Dr. J. Knaack of the Departments of Surgery and Pathology respectively, Royal Victoria Hospital, for generously providing parathyroid tissue for this study, and Dr. M. van der Rest of the Shriners Hospital, Montreal, Que., Canada, for performing the amino acid analysis of human parathyrin. This work was supported by grants MA-5775 and MT-1658 and a scholarship from the Medical Research Council of Canada, and by grant no. HDO4365 from the United States Public Health Service. D. G. and H. P. J. B. are recipients of scholarships from the Conseil de la Recherche en Santé du Québec.

References

- Bennett, H. P. J., Hudson, A. M., McMartin, C. & Purdon, G. E. (1977) *Biochem. J.* **168**, 9–13
- Bennett, H. P. J., Hudson, A. M., Kelly, L., McMartin, C. & Purdon, G. E. (1978) *Biochem. J.* **175**, 1139–1141
- Bennett, H. P. J., Browne, C. A. & Solomon, S. (1980a) *J. Liquid Chromatog.* **3**, 1353–1365
- Bennett, H. P. J., Browne, C. A., Brubaker, P. L. & Solomon, S. (1980b) in *Biological and Biomedical Applications of Liquid Chromatography* (Hawk, G. L., ed.), Marcel Dekker, New York and Basel, in the press
- Bennett, H. P. J., Browne, C. A., Goltzman, D. & Solomon, S. (1980c) *Proc. Am. Peptide Symp.* **6th** 121–124
- Berson, S. A. & Yalow, R. S. (1968) *J. Clin. Endocrinol. Metab.* **28**, 1037–1047
- Canterbury, J. D. & Reiss, E. (1972) *Proc. Soc. Exp. Biol. Med.* **140**, 1393–1398
- Chambers, D. J., Dunham, J., Zanelli, J. M., Parsons, J. A., Bitensky, L. & Chayen, J. (1978) *Clin. Endocrinol.* **9**, 375–379
- Goldsmith, R. S., Fruszyfer, J., Johnson, W. J., Fournier, A. E., Sizemore, G. W. & Arnaud, C. D. (1973) *J. Clin. Invest.* **52**, 173–180
- Goltzman, D., Callahan, E. N., Tregear, G. W. & Potts, J. T., Jr. (1975a) *Proc. Am. Peptide Symp.* **4th** 571–577
- Goltzman, D., Peytremann, A., Callahan, E., Tregear, G. W. & Potts, J. T., Jr. (1975b) *J. Biol. Chem.* **250**, 3199–3203
- Goltzman, D., Callahan, E. N., Tregear, G. W. & Potts, J. T., Jr. (1976a) *Biochemistry* **15**, 5076–5082
- Goltzman, D., Peytremann, A., Callahan, E. N., Segre, G. V. & Potts, J. T., Jr. (1976b) *J. Clin. Invest.* **57**, 8–9
- Goltzman, D., Callahan, E. N., Tregear, G. W. & Potts, J. T., Jr. (1978) *Endocrinology* **103**, 1352–1360
- Goltzman, D., Henderson, B. & Loveridge, N. (1980) *J. Clin. Invest.* **65**, 1309–1317
- Keutmann, H. T., Barling, P. M., Hendy, G. N., Segre, G. V., Niall, H. D., Aurbach, G. D., Potts, J. T., Jr. & O'Riordan, J. L. H. (1974) *Biochemistry* **13**, 1646–1652
- Keutmann, H. T., Hendy, G. N., Boehnert, M., O'Riordan, J. L. H. & Potts, J. T., Jr. (1978a) *J. Endocrinol.* **78**, 49–58
- Keutmann, H. T., Sauer, M. M., Hendy, G. N., O'Riordan, J. L. H. & Potts, J. T., Jr. (1978b) *Biochemistry* **17**, 5723–5729
- Lewis, R. V., Fallon, A., Stein, S., Gibson, K. D. & Udenfriend, S. (1980) *Anal. Biochem.* **104**, 153–159
- Parsons, J. A., Reit, B. & Robinson, C. J. (1973) *Endocrinology* **92**, 454–472
- Parsons, J. A., Rafferty, B., Gray, D., Reit, B., Zanelli, J. M., Keutmann, H. T., Tregear, G. W., Callahan, E. N. & Potts, J. T., Jr. (1975) in *Calcium-Regulating Hormones* (Talmage, R. V., Owen, M. & Parsons, J. A., eds.), pp. 33–39, Excerpta Medica, Amsterdam
- Segre, G. V., Habener, J. F., Powell, D., Tregear, G. W. & Potts, J. T., Jr. (1972) *J. Clin. Invest.* **51**, 3163–3172
- Silverman, R. & Yalow, R. S. (1973) *J. Clin. Invest.* **52**, 1958–1971
- Tregear, G. W., von Rietschoten, J., Greene, E., Keutmann, H. T., Niall, H. D., Reit, B., Parsons, J. A. & Potts, J. T., Jr. (1973) *Endocrinology* **93**, 1349–1353
- Wells, S. A., Jr., Ellis, G. J., Gunnells, J. C., Schneider, A. B. & Sherwood, L. M. (1976) *N. Engl. J. Med.* **295**, 57–62
- Zanelli, J. M. & Gaines-Das, R. E. (1980) *J. Endocrinol.* **86**, 291–304