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PURIFICATION AND ASSAY OF BOVINE PARATHYROID HORMONE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) has been used to purify a crude extract of bovine parathyroid glands, in a single run on an analytical column, to give a high yield of homogeneous material with full bioactivity in in vivo bioassay. Bovine parathyroid hormone (bPTH) prepared and purified by conventional procedures has been rapidly and quantitatively separated from its oxidation and other degradation products, from hormone fragments and from non-hormonal contaminants. Recovery of bPTH, monitored by region-specific immunoassays, in vivo bioassay and re-chromatography on HPLC was > 93%. The detection limit of the HPLC system, using endogenous tryptophan fluorescence, was 20 ng bPTH.

INTRODUCTION

Conventional methods for the purification and assay of extracted bovine parathyroid hormone (bPTH), reviewed in ref. 1, are associated with cumulative loss of up to 40% of the hormone during lengthy gel and ion-exchange

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chromatography and with costly, laborious and insensitive bioassay. We here report the use of a reversed-phase high-performance liquid chromatographic (HPLC) system, already exploited for the study of many other peptides in complex biological materials [2—5], to purify a crude bPTH extract in a single preparative run on an analytical column to give a high yield of homogeneous material with in vivo biological activity of 2300 IU/mg.

MATERIALS AND METHODS

Preparations

Eight bPTH materials studied by HPLC included two bulk preparations — crude trichloroacetic acid precipitated bPTH (TCA bPTH, batch 280178), and highly purified bPTH (bPTH, batch 100375), both prepared at the National Institute for Biological Standards and Control; four ampouled preparations — the WHO International Reference Preparation of Parathyroid Hormone, Bovine, for Immunoassay (IRP ibPTH, ampoule code 71/324), IRP bPTH for Bioassay (IRP bioPTH, ampoule code 67/342), Medical Research Council Clinical Reagent (MRC bPTH, code 72/286), and NIBSC Clinical Reagent (NIBSC bPTH, code 77/533). Ampouling was carried out according to procedures used for international biological standards [6]. Other materials included in the study were oxidized bPTH (batch 100375) containing less than 10% residual bioactivity by in vivo bioassay, a common contaminant believed to be bovine haemoglobin α-chain [7], synthetic bPTH fragment 1—34 and enzymatically cleaved fragment 53—84 (kindly supplied by Dr. J. Tregear and Dr. H. Keutmann).

HPLC methods

Gradient conditions and equipment used have been described previously [3, 4] and are indicated in the legend to Fig. 1. The system used ODS-Hypersil (Shandon Southern, Runcorn, Great Britain) in 150 × 4.6 mm I.D. stainless-steel columns. The primary solvent (in which samples were injected) was 0.155 *M* sodium chloride (pH 2.1) and components were separated at 35°C using a gradient of three linear segments, with acetonitrile as the secondary solvent, at a flow-rate of 1 ml/min. The eluate was monitored sequentially by UV absorbance at 215 nm and by endogenous tryptophan fluorescence (activation wavelength 225 nm, emission filter 340 nm). Isocratic separations were carried out at ambient temperature on Nucleosil 5 C8 (Macherey, Nagel & Co., Düren, G.F.R.) in 100 × 5 mm stainless-steel columns. The mobile phase was 0.155 *M* sodium chloride (pH 2.1)—acetonitrile (70:30) at a flow-rate of 0.5 ml/min [5]. Fractions were collected in narrow glass or polypropylene tubes containing 0.1 ml of 0.1% bovine serum albumin, snap-frozen, lyophilized and stored at −40°C until reconstituted in the appropriate buffer for immuno- or bioassay.

Radioimmunoassay

An overnight radioimmunoassay (RIA) system was used with ^{125}I-bPTH (batch 100375), and antisera Burroughs Wellcome 211/32 (NIBSC Code 76/507) (final dilution of 1/300,000 for intact and amino-region bPTH) and NIBSC/Wellcome 1127/21 (final dilution 1/300,000 for intact and carboxyl-region bPTH). Separation of bound and free fractions was achieved in 4 h using

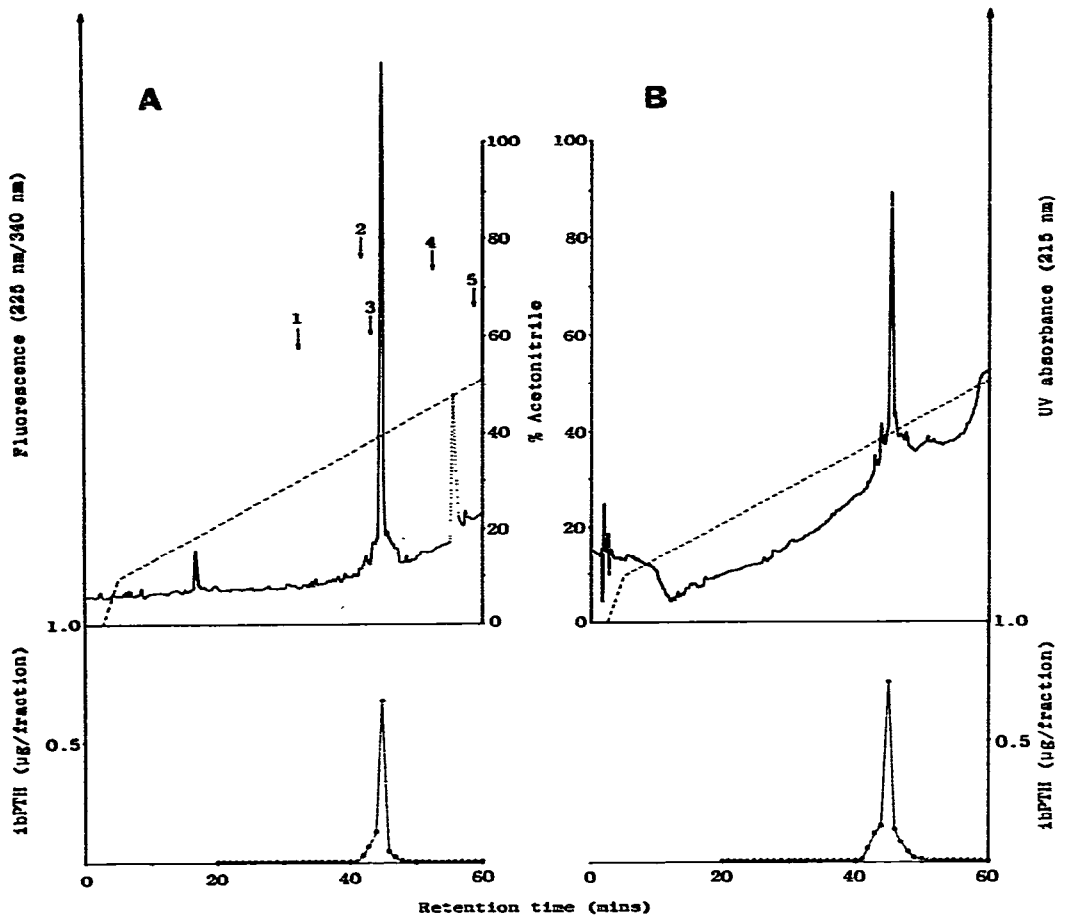

Fig. 1. Gradient elution HPLC of bovine parathyroid hormone on ODS-Hypersil. Separations were carried out at 35°C and constant flow-rate of 1 ml/min, with 0.155 *M* sodium chloride (pH 2.1) as primary solvent and acetonitrile as the organic modifier (see dashed line for gradient profile). (A) Fluorescence trace from highly purified bulk bPTH (100375) (the dotted peak represents a solvent impurity); (B) UV absorbance of an ampouled preparation (77/533). The corresponding profiles of immunoreactivity (ibPTH) in 1-ml eluent fractions are shown below each trace. The arrows on (A) indicate the retention times of (1) $bPTH_{53-84}$, (2) oxidised bPTH (100375), (3) $bPTH_{1-34}$, (4) human serum albumin and (5) bovine haemoglobin α-chain.

a polyethylene-glycol-accelerated second antibody precipitation. The detection limit of intact bPTH in both RIA systems, using IRP ibPTH as standard and assuming 1 µg bPTH per ampoule was 0.9 ng/ml; 50% displacement was 9 ng/ml. An aliquot of the solution loaded on to the HPLC columns was included in serial dilution in the RIA as the standard curve for estimation of recovery. HPLC fractions were assayed at 2—4 dilutions to screen for immunochemical heterogeneity.

In vivo bioassay

The intact chick, 1-h acute hypercalcaemia [8] assay was carried out, and the

doses were injected intravenously. The bioassay house standard was highly purified ampouled MRC Clinical Reagent (code 72/286) and results expressed in International Units defined by the IRP bio-PTH standard. It is generally accepted that bulk highly purified bPTH can be expected to have an in vivo biological activity of approximately 2500 IU/mg [1].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in 6 *M* urea was carried out according to the procedure of Panyim and Chalkley [9]. Samples from HPLC were loaded in up to 50 μl of eluent after evaporation of acetonitrile.

RESULTS AND DISCUSSION

Chromatograms for carrier-free highly purified bPTH and an ampouled preparation of highly purified bPTH (code 77/533) together with profiles of immunoreactivity (obtained with the RIA for intact and amino-region bPTH) are shown in Fig. 1A and B. The retention times for oxidized bPTH, haemoglobin α-chain and the 1—34 and 53—84 bPTH fragments are marked on Fig. 1A for reference. In all preparations there were indications of a shoulder at each side of the main peak. Isohormones have been described [10] and it is possible that the HPLC systems used may partially resolve these. A consistent pattern of small peaks eluting close to the main component, present in bulk and ampouled preparations (77/533 and 72/286) of highly purified bPTH, was seen in both UV and fluorescence traces. Relative proportions of the peaks varied but no single minor peak contained more than 10% of total UV absorbance and 5% total immunoreactivity. More than 90% of UV absorbance and tryptophan fluorescence was associated with the major peak for the IRP ibPTH (code 71/324), previously characterized as bPTH isohormone I [10]. Immediate reinjection of the major HPLC peak fraction of highly purified bPTH 100375, after dilution in primary solvent, yielded only a single peak of identical size and gave a single band on polyacrylamide gel electrophoresis in 6 *M* urea (Fig. 2). Minor components in bPTH ampoule 77/533 were more clearly seen under isocratic conditions (Fig. 3). Rechromatography of the main peak fraction after it had been lyophilized under laboratory conditions showed about 12% of the total UV absorbance appearing as an earlier peak. Controlled oxidation of highly purified bPTH with hydrogen peroxide yielded a series of peaks whose retention times corresponded to some of the minor components seen in the preparations tested. However, more vigorously oxidized bPTH reference material was not retained under the isocratic conditions, although eluting only 3 min before bPTH on gradient systems (Fig. 1A).

Analysis of the IRP bioPTH (ampoule code 67/342), which had been prepared by TCA precipitation and which was known to give anomalous results in some in vivo and in vitro bioassay systems, showed an atypical gradient elution chromatogram (Fig. 4) when compared with other bPTH preparations. The peak identified as bPTH by its retention time and immunoreactivity was superimposed on a broad fluorescence and UV absorbing background peak. It is not known whether this was an atypical batch of TCA bPTH (the bulk TCA bPTH, prepared in 1966, is no longer available) or whether artifacts had arisen at one or more stages of the ampouling procedure.

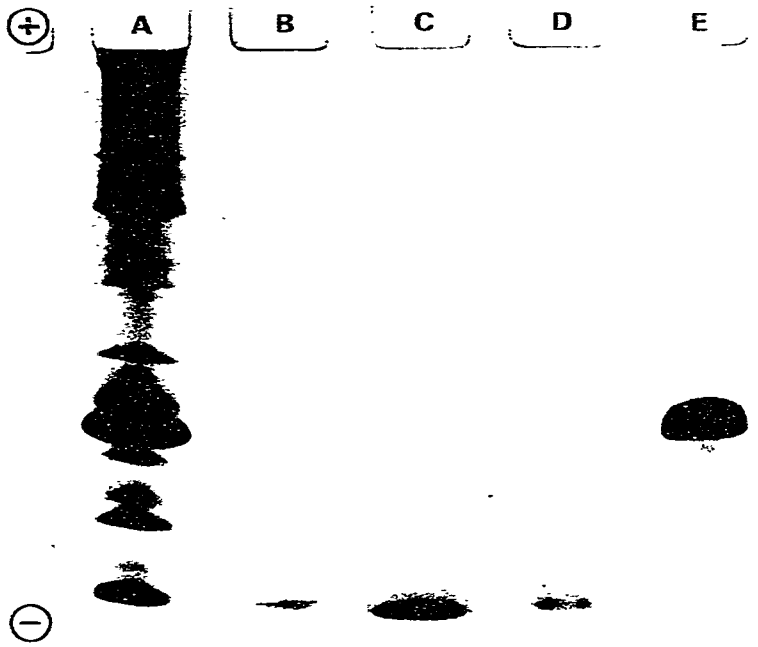

Fig. 2. Polyacrylamide gel electrophoresis of bulk and HPLC-purified bPTH preparations. Separations were carried out on 15% gels with 6 *M* urea using 0.9 *N* acetic acid (pH 2.5). (A) TCA precipitate of parathyroid glands (280178) containing approximately 2—3% bPTH; (B) bPTH purified from TCA precipitate (280178) by HPLC; (C) highly purified bPTH (100375) before HPLC; (D) bPTH (100375) after HPLC; (E) bovine haemoglobin α-chain from parathyroid glands.

A chromatogram and an immunoreactivity profile for bulk crude TCA bPTH (280178) are shown in Fig. 5. More than 79% of the total immunoreactivity corresponded to the fluorescent peak at 45 min and comparison of peak height with that given by highly purified bPTH (bulk batch 100375 used as HPLC reference) indicated a bPTH content of 30 μg/mg, in good agreement with the original bioassay estimate of potency of 50 IU/mg (approximately 2% pure), and also with measurement of total recovered immunoreactivity (35 μg). The practical detection limit based on tryptophan fluorescence was 20 ng with a linear response between 20 ng and at least 12.5 μg, and a precision for replicate assays of 2.2% (C.V., n = 7). After preparative HPLC (10-mg load), which gave an identical profile to that shown in Fig. 5, the bPTH peak yielded material on which a bioassay estimate of 2300 IU/mg (95% confidence limits 1700—3100 IU/mg) was obtained, and which gave a single band on PAGE (Fig. 2).

There was no significant indication from results of the two RIA systems of immunochemical heterogeneity in the major (or minor) peaks after HPLC of two crude and three highly purified preparations. As, furthermore, none of the minor HPLC peaks resolved by isocratic chromatography (Fig. 3) had retention times equal to those of fragments of known structure, it seems likely that heterogeneity revealed by this HPLC system was not due to the presence of hormonal fragments.

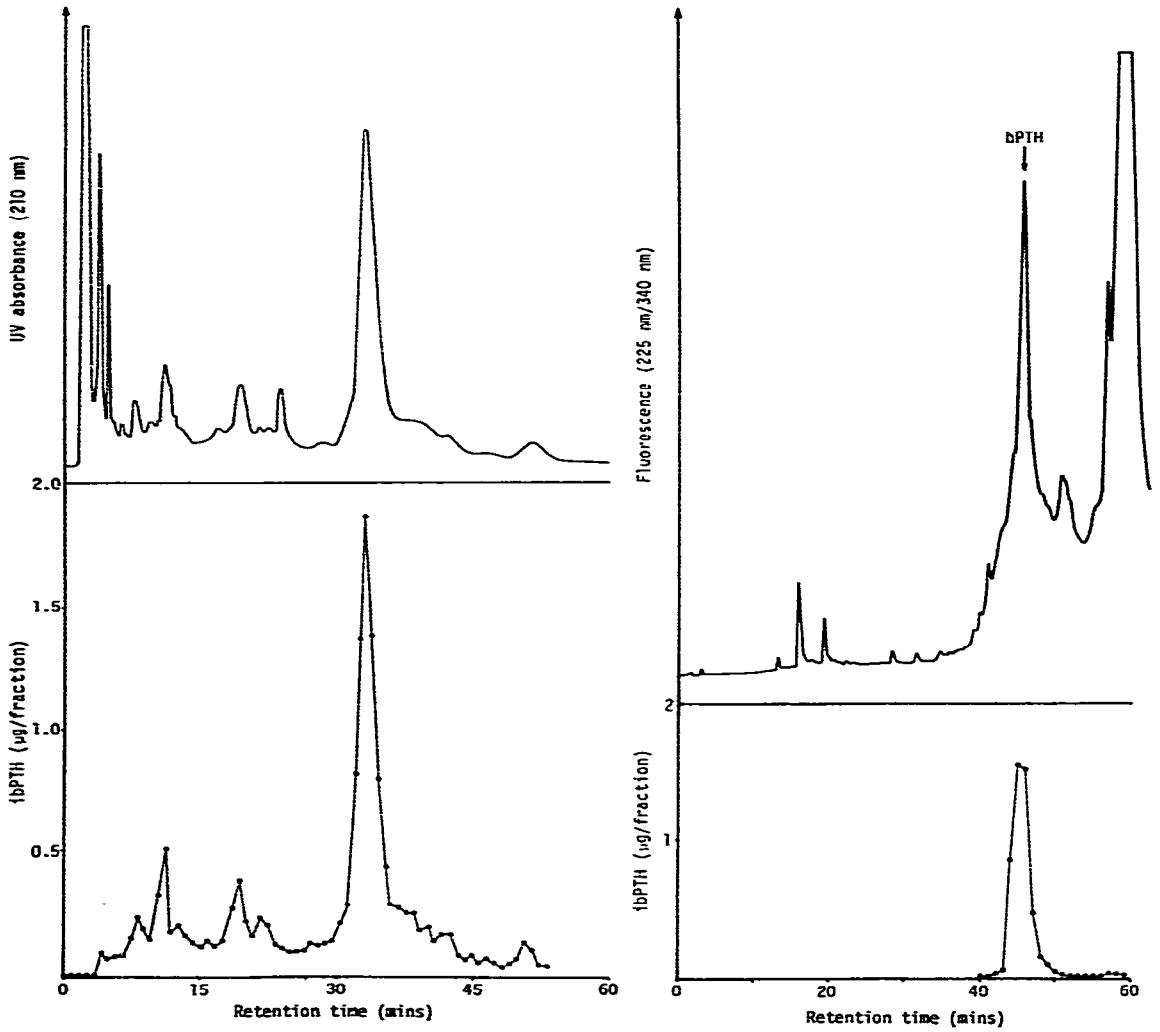

Fig. 3. Isocratic HPLC of an ampouled bovine parathyroid hormone preparation (77/533) on Nucleosil 5 C8. Separations were obtained at ambient temperature and a flow-rate of 0.5 ml/min using a mobile phase of 0.155 *M* sodium chloride (pH 2.1)—acetonitrile (70:30). The upper trace shows the UV absorbance (210 nm) (cf. Fig. 1B) with the corresponding immunoreactivity profile in 200-μl eluent aliquots below.

Fig. 4. Gradient elution HPLC of ampouled International Reference Preparation for Bioassay (bioPTH 67/342) prepared by TCA precipitation. Chromatographic conditions were as given in Fig. 1. The fluorescence chromatograms and the corresponding immunoreactivity in 1-min eluent fractions are shown. The retention time of a bPTH reference standard (100375) is indicated for comparison (cf. Fig. 1). Note that the bPTH 67/342 gave a relatively broad fluorescent/immunoreactive peak compared with other preparations using gradient elution.

Retention of bPTH on the reversed-phase supports used under the conditions described is expected to depend on the content of hydrophobic residues; thus reversed-phase HPLC is complementary to conventional evaluation of bPTH preparations by molecular size or charge. Oxidation of methionine residues will reduce hydrophobicity and oxidation of bPTH results in the appearance of

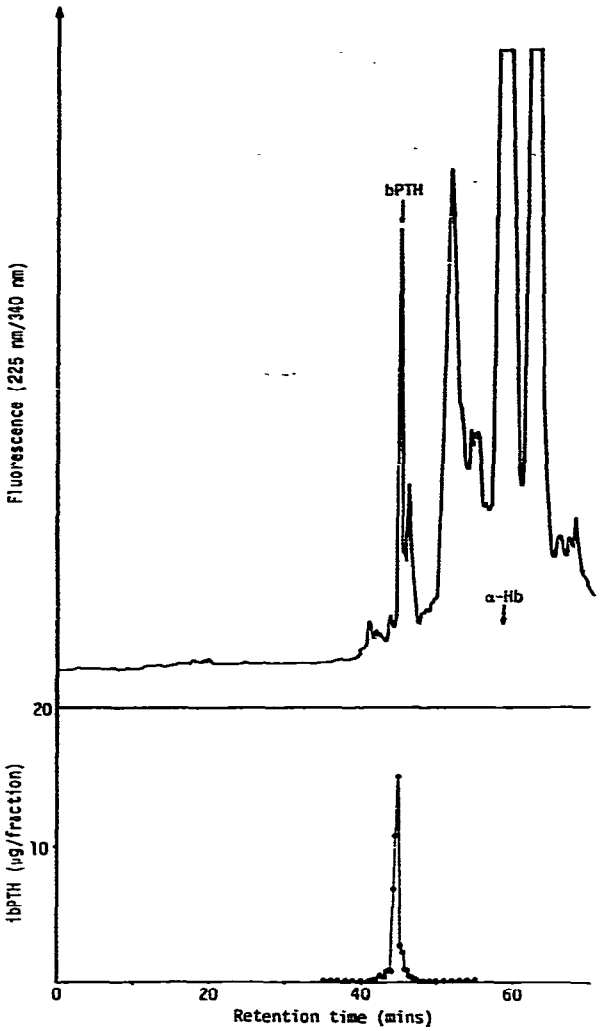

Fig. 5. Gradient elution HPLC of 1 mg of bulk crude TCA precipitated bPTH (280178). Chromatographic conditions were as given in Fig. 1. The fluorescence profile of this material is shown, together with the associated immunoreactivity in 18-sec eluent fractions. The retention times of highly purified bPTH (100375) and of haemoglobin α-chain are also indicated. The total amount of immunoreactive material recovered (35 μg) corresponded well with the relative peak height on the fluorescence trace (equivalent to 30 μg bPTH 100375).

peaks with decreased retention times. Some minor peaks eluting before the main peak in bPTH are probably attributable to different oxidation products arising during isolation and purification and, possibly, during ampouling procedures. The nature of the minor peaks eluting after the main peak is unknown. However, it has been suggested that deamidation of glutamine and asparagine residues may occur during the acid conditions of extraction and conventional purification of bPTH [1, 11]. Such deamidation products might be expected to have longer retention times than the native molecule, as found for insulin [12, 13].

In summary, reversed-phase HPLC under gradient conditions, complemented by isocratic chromatography for detailed examination of closely associated components has been shown to be (1) a rapid, reproducible, specific, sensitive and quantitative system for the assay of bPTH in crude and highly purified bulk materials and in preparations ampouled with carrier substances; (2) a high-resolution system for the assessment of heterogeneity of highly purified bPTH by the separation of bPTH from its oxidation and other degradation products, small peptide fragments, a common contaminant (bovine haemoglobin α-chain) and from added carrier proteins (serum albumins); (3) a rapid and complete system for high-yield purification of homogeneous bPTH with high biological activity from a crude extract. Since individual degradation products and the enzymatically cleaved 53—84 fragment are readily resolved under appropriate conditions, their recovery in high yield in highly purified form is also possible. Reversed-phase HPLC systems such as those described in this report are likely to have immediate application to the resolution and recovery (for analysis in other systems) of other highly purified hormonal components synthesized and released into culture medium by parathyroid cells maintained in vitro [14] or of specific bPTH enzymatic cleavage products, such as those produced by parathyroid and liver cathepsin B, an enzyme postulated to play a major role in peripheral inactivation as well as in the glandular regulation of secretion of the parathyroid hormone [15].

Reversed-phase HPLC methods are also being used to study human PTH (hPTH) [16]. We are now using the method described herein, in conjunction with an ultra-sensitive cytochemical bioassay for hPTH [17] and region-specific hPTH immunoassays, to separate components in partially and highly purified extracts of human parathyroid adenomata, and in biological fluids derived from such tissue maintained in vitro [18].

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REFERENCES

1 H.T. Keutmann, Clinics in Endocrinology and Metabolism, 3 (1974) 173.
2 H.P. Bennett, A.M. Hudson, C. McMartin and G.E. Purdon, Biochem. J., 168 (1977) 9.
3 M.J. O'Hare and E.C. Nice, J. Chromatogr., 171 (1979) 209.
4 E.C. Nice, M. Capp and M.J. O'Hare, J. Chromatogr., 185 (1979) 413.
5 P.H. Corran, B. Rafferty and J.M. Zanelli, Fifth International Conference on Analytical Chemistry, Lancaster, Great Britain, 1980.
6 World Health Organisation Expert Committee on Biological Standardization. 29th Report, Annex 4, WHO Tech. Rep. Ser., 626 (1978) 108.
7 H.T. Keutmann, personal communication.

8 J.A. Parsons, B. Reit and C.J. Robinson, Endocrinology, 92 (1973) 454.
9 S. Panyim and R. Chalkley, Arch. Biochem. Biophys., 130 (1969) 337.
10 H.T. Keutmann, G.D. Aurbach, B.F. Dawson, H.D. Niall, L.J. Deftos and J.T. Potts, Biochemistry, 10 (1971) 2779.
11 H.B. Brewer, T. Fairwell, W. Rittell, T. Littledike and C.D. Arnaud, Amer. J. Med., 56 (1974) 759.
12 U. Damgaard and J. Markussen, Horm. Metab. Res., 11 (1979) 580.
13 A. Dinner and L. Lorenz, Anal. Chem., 51 (1979) 1872.
14 E.M. Brown, D.G. Gardner and G.D. Aurbach, Endocrinology, 106 (1980) 133.
15 R.R. MacGregor, J.W. Hamilton, G.N. Kent, R.E. Shofstall and D.V. Cohn, J. Biol. Chem., 254 (1979) 4428.
16 H.P.J. Bennett, D. Goltzman, C.A. Browne and S. Solomon, Proc. IV International Congress on Endocrinology, Melbourne, 1980, p. 343.
17 D.J. Chambers, J. Dunham, J.M. Zanelli, J.A. Parsons, L. Bitensky and J. Chayen, Clin. Endocrinol., 9 (1978) 375.
18 J.M. Zanelli and R.E. Gaines-Das, VII International Conference on Calcium Regulating Hormones, Estes Park, CO, 1980, Abstract 149.