RADIOAKTIVE ISOTOPE IN KLINIK UND FORSCHUNG

13. Band

1. Teil

Gasteiner
Internationales Symposium
1978

Herausgegeben
von Rudolf Höfer
unter Mitarbeit von
Helmar Bergmann

Verlag
H. Egermann

ISBN 3-900287-01-5

Radioaktive Isotope in Klinik und Forschung
veranstaltet von der Nuclearmedizinischen Abteilung
der II. Medizinischen Universitätsklinik,
Leiter: Prof. Dr. R. Höfer, A-1090 Wien, Garnisongasse 13

Printed by: Egermann Druckereigesellschaft m. b. H. & Co. KG.,
A-1170 Vienna

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RAPID AND SENSITIVE RADIOIMMUNOASSAYS FOR HUMAN PARATHYRIN

W.G. Wood, I. Marschner and P.C. Scriba

INTRODUCTION

The main disadvantage in the radioimmunoassay (RIA) of human parathyrin (hPTH) is the long incubation time supposedly required to achieve the sensitivity needed in measuring hPTH in normals (1), (2), (4), (9). To minimise the time between receipt of the blood and the arrival of results on the doctor's desk, studies were undertaken to minimise the assay time without reducing the sensitivity in the assay of carboxy-regional peptides of hPTH (C-hPTH). By optimising the assay, the incubation time was reduced from 7 days in the original assay (9) to 6 or 24 hours without influencing the clinical value of the results.

MATERIALS AND METHODS

a) Antibodies. The antibodies used were obtained from Drs. HESCH and HEHRMANN in Hannover (FRG) and were raised in sheep immunised with a mixture of bovine and porcine parathyrin (bPTH, pPTH). The code numbers allotted to the antibodies (Ab) were S-469 VI and S-478 VI. Ab S-469 VI was used at an initial dilution (i.d.) 1:20 000 and S-478 VI at an i.d. of 1:2400.

b) Standards. bPTH was obtained from Inolex Chicago, Lot No. 1508 B 002 for standards and Lot No. 1515 A 001 for labelling. "C-hPTH-standards" were obtained from a patient with secondary hyperparathyrinaemia (2. HPT) and were calibrated against the S-469 VI Ab.

All standards were made up in parathyrin free serum (PTHFS) obtained from parathyroidectomised patients.

Tracer was prepared by a modified form of the Chloramine-T-method of GREENWOOD et al. (7). 10 µg bPTH (1 µg/µl in 0.1 M HCl) was reacted with 10 µl Chloramine-T (8.9 mM), 20 ul $Na^{125}I$ (37.2 MBq) and 100 µl phosphate buffer (0.5 M, pH 7.65) for 30 sec. when the reaction was stopped with 100 µl $Na_2S_2O_5$ (6.6 mM). The mixture was then transferred to a 9 x 1 cm Sephadex G-10 column and eluted off using 0.05 M barbital buffer, pH 7.8, containing 1% human serum albumin (HSA) and 12.5 antiplasmin units (APU) of Aprotenin/ml (Trasylol®-Bayer), (Buffer 2).

The protein peak containing ^{125}I-bPTH was then transferred to a 130 x 2.5 cm Ultrogel AcA-54 column and eluted off at 4° C with 0.5 M ammonium acetate buffer (pH 4.5) containing 0.01% merthiolate (Buffer 3). The fractions containing the radioactive peaks were tested for immunoreactivity and those with the highest maximum binding (B_o) and lowest unspecific binding (N) diluted to an end radioactivity of 10 KBq ml. The tracer was useable for more than 3 months without appreciable change in the binding characteristics to the antibody. Both Ab and tracer were diluted with Buffer 2.

c) Assays.

(I) Original Assay - S-469 50CL. This was the original assay used by HEHRMANN et al. (9) in which 50 µl serum and 200 µl S-469 VI (i.d. 1:20 000) were preincubated for 4 days at 4° C. 100 µl tracer (50 Bq) were added and a further 3 day incubation carried out at 4° C. 1000 µl dextran-charcoal (2.5 g Dextran T-70 (Pharmacia) + 25 g Norit A in 1000 ml Buffer 2) was added to the tubes which were then centrifuged for 15 minutes, after which the supernatant (1000 µl) was transferred to a clean tube, stoppered and counted for 5 minutes. Range of the standard curve was 0.19 - 12.5 ng bPTH/ml.

(II) Modified Long assay - S-469 50L. This assay was identical with (I) above except that 170 Bq tracer was used, and separation of bound and free hormone was with 1000 ul 12% polyethylene glycol (PEG) in 0.18 M NaCl. After centrifugation and aspiration of the supernatant, the precipitate was counted for 2 minutes. In both (I) and (II) B_0 was set up in 10 tubes and each standard/serum in 5 tubes.

(III) Short Assay - S-478 90Q. The difference here was the use of Ab S-478 VI (i.d. 1:2400), 90 µl standard/serum instead of 50 µl, preincubation of 18 hr, and incubation with tracer (330 Bq) 6 hr. Separation of bound and free hormone was by 12% PEG. Here B_0 was set up in 6 tubes and each standard or serum in 3 tubes.

(IV) Superquick Assay - S-478 90SQ. Here a standard curve was set up using C-hPTH standards (0.15 - 10 ng eq/ml) and the assay consisted of a single 6 hr incubation with serum (90 µl) Ab (200 µl S-478 VI i.d. 1:2400) and tracer (100 µl = 500 Bq) and PEG separation. Tubes were counted for 60 - 90 sec.

d) Kinetic Studies.

Kinetic studies were carried out using Ab S-478 VI. Human sera with 1 and 3 ng eq/ml and bPTH standards of 1 and 3 ng/ml together with PTHFS as a zero standard were compared over a 7 - 10 day incubation period - with and without 18 hr-preincubation.

e) Establishment of normal range.

Normal ranges were established for the S-469 50L and S-478 90Q assays together with correlations between the two with a comparison of a normal collective, patients with calcium metabolism disturbances not due to parathyroid changes and patients with primary and secondary hyperparathyrinaemia (1. and 2.HPT).

RESULTS

a) Tracer

Fig. 1a shows the elution profile of the tracer on Sephadex G-10 and Fig. 1 b of the ^{125}I-bPTH peak from the Sephadex G-10 on Ultrogel AcA-54. In Fig. 1 b the radioactive peaks are seen together with B_0 and N in the main peaks. The second main peak is probably the alpha-chain of haemoglobin and it shows no reaction with either the C-regional antibodies S-469 VI, S-478 VI or the European Parathyroid Study

Group (EPSG) anti-1-34 hPTH. With this preparation no "damage" was to be seen.The tracer could be used for longer than 3 months.

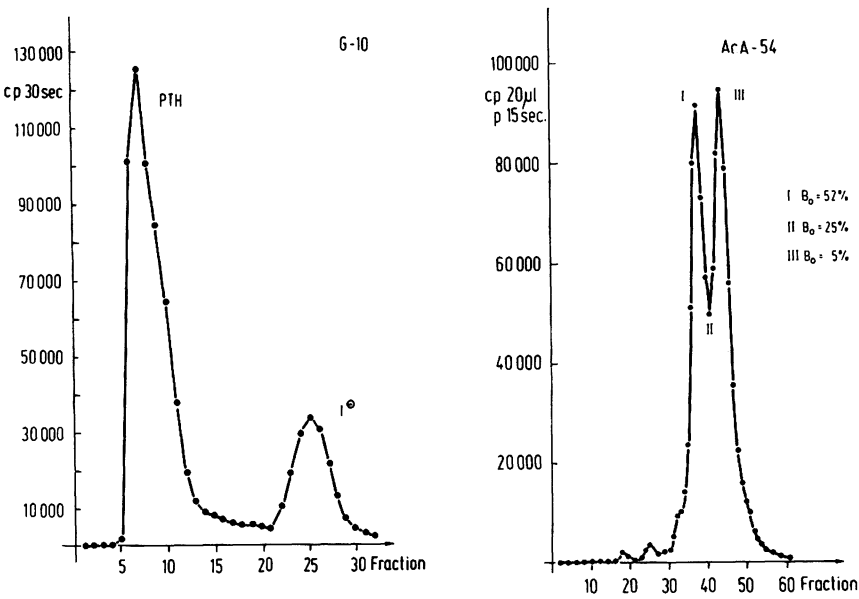

Fig. 1 (a) left : Elution profile of ^{125}I-bPTH mixture from iodination on Sephadex G-10. Fractions contain 0.55 ml - column 9x1 cm.

(b) right: Elution profile of ^{125}I-PTH peak on Ultrogel AcA-54. I, II and III show maximum binding (B_o) of immunoreactive bPTH in the main peaks after 6 hr incubation at 4° C with Ab S-469 VI i.d. 1:1000. Fractions contained 4.55 ml.

b) Assays

In the S-478 90Q assay (as well as the S-469 50Q assay) the "hook-effect" (11) was abolished with the increase in tracer. Fig. 2 shows the normal range of S-469 50L and S-478 90Q in 47 volunteers and the results of 90 patients without HPT and with 1. and 2. HPT.

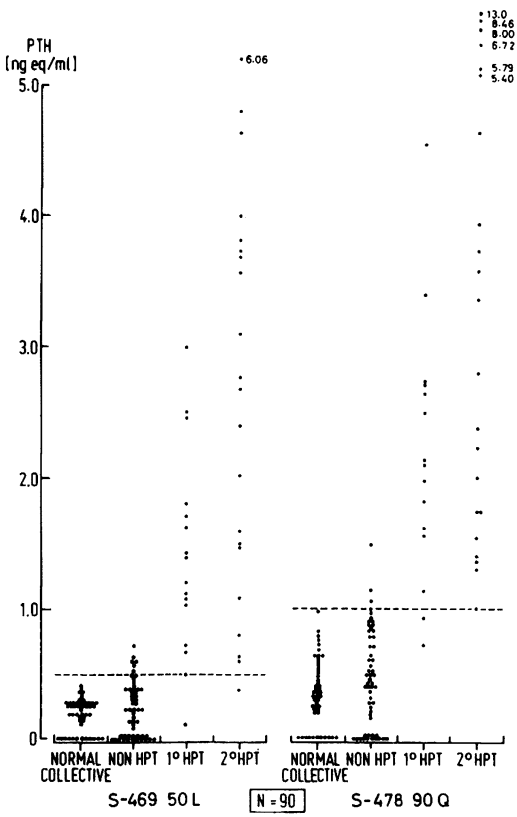

Fig. 2: Normal ranges (upper limits shown by dotted lines) in 47 normocalcaemic volunteers and 90 patients with calcium metabolism disturbances not due to HPT (Non-HPT) and primary and secondary HPT (1° HPT, 2° HPT).

Correlation between 109 patients measured in both assays gave the following values - correlation coefficient $r = 0.872$ and for the regression equation $y = a + bx$, $a = 0.323$, $b = 1.217$.

Table 1 shows the main characteristics of the S-469 5OL and S-478 9OQ assays. Table 2 shows results of a catheter study of the neck veins in a patient with suspected 2. HPT using S-469 5OL, S-478 9OQ and S-478 9OSQ assays.

The superquick assay S-478 9OSQ was only intended for emergency and was not introduced into routine use, although one can see that the clinical value for localisation of an adenoma is as good as the other assays when compared with the values of the control sera.

Table 1: Main characteristics of S-469 5OL and S-478 9OQ assays.

	S-469 5OL	S-478 9OQ
1) Ab initial dilution	1:20 000	1:2400
2) Incubation times		
(a) preincubation	96	18
(b) after tracer addition	72	6
3) 100 . $(B_o-N)/T$	30	10
4) Normal range ng eq/ml	up to 0.5	up to 1.0
5) Sensitivity ng bPTH/ml	0.28 ± 0.13 (n=10)	0.28 ± 0.08 (n=12)
6) 50% intercept ng bPTH/ml	2.4 ± 0.4 (n=10)	2.5 ± 0.2 (n=12)
7) Tracer/tube Bq	170	330

Table 2: Results of a catheter study of the veins in the thyroid region of a patient with suspected 2° HPT.

	Assay - ng eq/ml		
Control sera	S-469 5OL	S-478 9OQ	S-478 9OSQ
1	0.41	0.21	0.17
2	0.79	0.55	0.53
3	3.04	3.35	4.80
Catheter location			
r.int.jug.v.upp.	0.90	1.27	1.28
low.	0.95	1.38	1.21
l.int.jug.v.upp.	0.75	1.42	1.46
low.	1.05	1.26	1.39
thyroid ima v.	4.89	5.98	5.24
int.thyroid v.	4.05	5.96	4.32
l.subclavian v.	1.16	1.42	1.61

c) Kinetics

Fig. 3a shows the binding kinetics (Ab S-478 VI) over 7 days of human and bovine PTH with the ^{125}I-bPTH tracer, with 18 hr preincubation before tracer addition. Fig. 3 b shows the same data but without the preincubation prior to tracer addition. Here the difference in the binding of hPTH and bPTH is plain to see by the difference in the amount of tracer bound - especially in the initial phases, and shows why the S-478 90 SQ assay only works with a C-hPTH standard curve.

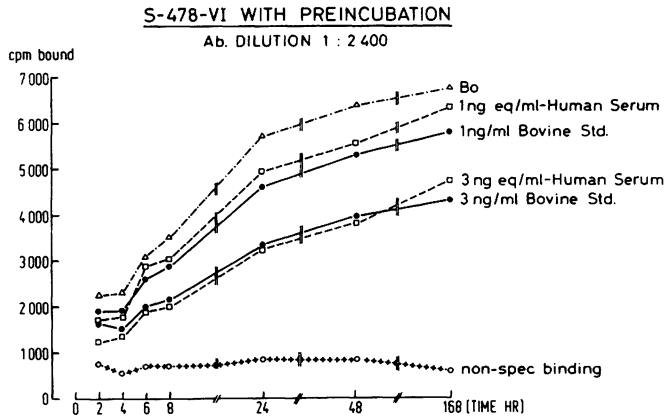

Fig. 3 a : Kinetics of Ab S-478 VI i.d. 1:2400 over a 168 hr period at 4° C. Tracer given after 18 hr preincubation of serum and antibody. Binding expressed as cpm bound (Total counts = 22000 cpm).

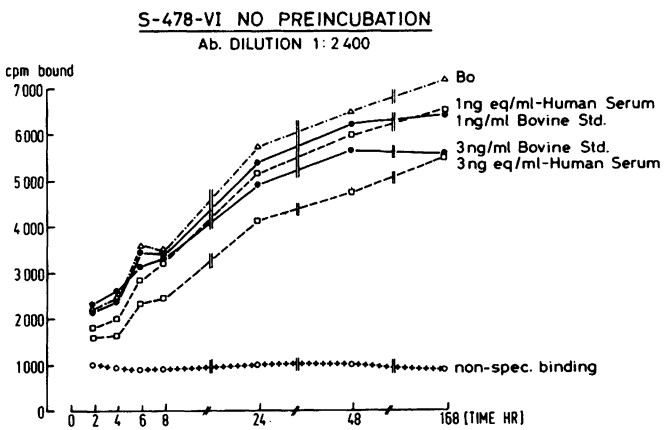

Fig. 3 b : As for Fig. 3 a, but without 18 hr preincubation step.

The circulating forms of hPTH are so varied (3), (5), (6), (8), (12) that different assays are needed to locate all the peptides present in the blood. The antibody used here (S-478 VI) recognises both C-regional peptides as well as the intact 1-84 hPTH molecule. This has been demonstrated in this laboratory and elsewhere (9), (10) by the fact that S-478 VI shows similar sloped dose response curves with the MRC 75/549 1-84 hPTH reference preparation as well as the tissue culture preparations P2 (1-84 hPTH) and P4 (C-regional hPTH) (of MONTZ in Hamburg). The fact that P2 and P4 show identical dilution curves (WOOD, 1977, unpublished data) demonstrates that Ab S-478 VI does not differentiate between 1-84 hPTH and C-regional fragments. Ab S-478 VI does not react with N-regional peptides (9).

Attempts to use anything except PTH-free serum in which to dissolve the standards were abandoned as the dose response curves did not run parallel to the PTHFS curves. Systems tried included serum from parathyroidectomised dogs, horses, "stripped-serum" and a synthetic serum-like solution of albumin and globulins in Ringer solution.
Similarly, attempts to use Ab S-469 VI in a short assay were quickly abandoned as the results were approximately 3-times higher than those from the S-469 50L assay, although the correlation coefficient was good ($r = 0.963$, $n=81$).

The use of PEG has its drawbacks in as much as it is serum globulin dependant. This means that all cases of disproteinaemia and dialysis patient sera as well as those with total protein outside the range 6-8 g/dl must be run with an unspecific binding. This accounts for about 10% of all patients in this laboratory.

The results show that despite the drastic shortening of the assay times, together with new labelling and separation techniques, the shorter assays give results which are just as sensitive and reproducible. The difference in reaction rates between bPTH and hPTH and Ab S-478 VI when no pre-incubation has been made, has been used in developing a same day-assay (S-478 90SQ) in which the clinician can receive the results of emergency cases of suspected HPT on the same day. By combining this with a catheter study of the neck-veins, the surgeon can know the most likely situation of an adenoma or hyperplastic parathyroid gland.

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Abstract

Radioimmunoassays for the carboxy-regional peptides of human parathyrin (hPTH) have been developed to give results within either 8 or 30 hr after receipt of blood, as compared with several days in the original method using the same antibodies (S-469VI, S-478VI). The achievement of such short assay times is due to a modification of the WIDE technique and the different binding between bovine and human parathyrin to the antibodies, raised in sheep against a mixture of porcine and bovine parathyrin. The results of these short assays have the same clinical value as the longer assays in differentiating between normals and patients suffering from hyperparathyrinaemia (HPT). The value of these assays is the rapid answer to clinicians and surgeons' questions as to whether a patient has HPT. By combination of the 8 hr-assay with a catheter study of the veins of the thyroid region, the surgeon can know on the same day not only whether the patient has HPT, but also the probable location of an adenoma if present, thus facilitating his task.

Extrait

On a développé des dosages radioimmunologiques pour les peptides carboxy régionales du parathyrin humain (PTH), qui donnent des résultats soit 8 heures, soit 30 heures après la prise du sang. Vis-à-vis de la méthode originale qui applique les mêmes anticorps S-469VI/S-478VI, on obtient ainsi une économie de temps de 4 jours. De tels essais brefs sont devenus possibles par la modification de la technique de WIDE; en outre on a exploité la différente liaison du parathyrin bovin et du parathyrin humain avec les anticorps, qui ont été gagnés dans des moutons par immunisation avec le parathyrin bovin et parathyrin porcin. Les résultats de ces essais brefs ont la même importance clinique que les essais longs pour différencier entre les patients à parathyrin normal et patients à hyperparathyrinémie (HPT). L'avantage de ces essais est la réponse rapide à la question des cliniciens, si un HPT est présent. Le chirurgien peut apprendre la localisation possible d'un adénome le même jour par la combinaison de l'essai de 8 heures avec l'étude des PTH-taux aux veines du cou.

Auszug

Es wurden Radioimmunoassays für die Carboxy-regionalen Peptide des humanen Parathyrins entwickelt, die Ergebnisse entweder 8 Stunden oder 30 Stunden nach Blutabnahme liefern. Gegenüber der Originalmethode, die die gleichen Antikörper S-469VI/S-478VI verwendet, wurde so eine Zeitersparnis von über 4 Tagen erreicht. Solche Kurzassays wurden möglich durch die Modifizierung der Wide-Technik. Ausgenutzt wird weiterhin die unterschiedliche Bindung des bovinen und des humanen Parathyrins mit den Antikörpern, die in Schafen durch Immunisierung mit bovinem und porcinem Parathyrin gewonnen wurden. Die Ergebnisse dieser Kurzassays haben die gleiche klinische Bedeutung wie die des Langassays zur Differenzierung zwischen Patienten mit normalem Parathyrin und solchen mit Hyperparathyrinämie (HPT). Der Vorteil dieser Assays ist die schnelle Antwort auf die Frage der Kliniker, ob eine HPT vorliegt. Bei der Kombination des 8 Stunden-Assays mit einer Katheterstudie der Halsvenen kann der Chirurg noch am gleichen Tag die mögliche Lage eines Adenoms erfahren.

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