REVISTA ESPAÑOLA DE FISIOLOGIA. 35, 21-28. 1979

# Standardization and Clinical Application of a Radioimmunoassay for Human Calcitonin

S. Prieto *, Amparo Pérez-Gutiérrez and J. Tamarit

Cátedra II de Fisiología
Facultad de Medicina
Universidad Complutense
Madrid - 3

(Received on February, 9, 1978)

S. PRIETO, A. PEREZ-GUTIERREZ and J. TAMARIT. *Standardization and Clinical Application of a Radioimmunoassay for Human Calcitonin.* Rev. esp. Fisiol., 35, 21-28. 1979.

A radioimmunoassay for the measurement of Human Calcitonin (HCT) is described. Serum levels of HCT in normal subjects and in individuals under different pathological conditions have been studied with this method. HCT labelling is performed following the chloramine T method of HUNTER and GREENWOOD. Adding successively Quso G-32 (a finely powdered silica) and an anion exchange resin (AGI-X10 resin), to the tracer, reduces both the damaged fraction and the free isotope which originate during storage. Purification of the labelled hormone is carried out through a Sephadex G-50 gel column. Sera stored at —20° C preserves its immunoreactivity up to 4 months after extraction. The mean basal HCT levels in 110 fasting normal persons is 277 ± 123 pg/ml (undetectable 8.28 %). No significant correlation between HCT levels and various serum ions has been observed. Basal HCT values in seven patients with medullary thyroid carcinoma (MTC), oscillated between 5 and 110 ng/ml, while in six other patients with non medullary thyroid carcinoma the values remained within normal range. Both a calcium infusion and a pentagastrin injection are used to stimulate HCT secretion.

The increase of HCT basal levels produced by the latter in normal controls and in patients with MTC, is faster and more intense. Calcium infusion produced a significant correlation between calcium and the increased HCT levels only in patients affected with MTC.

The Human Calcitonin (HCT) is a small peptide with a single chain of 32 aminoacids (11). It is released from the thyroid gland (2, 16) and found in peripheral blood of normal persons at levels lower than 1 ng/ml. Hence, only saturation analysis methods, like the Radioimmunoassay, will allow us to measure it.

In this paper a modification of methods previously described in the literature (1, 6, 12) is presented. Using this RIA-meth-

* S. PRIETO is recipient of a fellowship from «Plan de Formación de Personal Investigador. Ministerio de Educación y Ciencia». Spain.

od, HCT serum levels are measured in normal subjects, patients with different calcium metabolic disorders, and patients with thyroid tumors.

## Materials and Methods

*Labelling.* Synthetic HCT (Ciba-Geigy Pharm) diluted with 1 mM HCl and kept stored at —20° C, is used as standard. Labelling is performed at room temperature following the Chloramine T method of HUNTER and GREENWOOD (9, 10). Mixing of reagents is done by sucking with a nylon catheter (Portex-1, Portland Plastics. Hythe Kent, England) connected to a 0.1 ml Hamilton microsyringe, successively: 25 $\mu$l of 0.2 M, pH 7.5 phosphate buffer (labelling buffer), 20 $\mu$l of Chloramine T (1 mg/ml) and 15 $\mu$l of (1.5 mCi) of $^{125}$I (Radiochemical Centre Amersham). The catheter content is added to 2 $\mu$g of HCT dissolved in 20 $\mu$l of labelling buffer. After mixing for 30 seconds, reaction is stopped by adding 50 $\mu$l of sodium metabisulfite (2.5 mg/ml). One hundred microliter of potassium iodide (10 mg/ml) and 0.1 ml of 1 % bovine seroalbumin (BSA) are then successively added to the vial. All reagents are dissolved in labelling buffer. After this porcedure about 60 % of the radioactivity is trichloroacetic acid or talc precipitable.

To remove the damaged hormone, 5 mg of Quso G-32 (microfine granules of precipitated silica, Philadelphia Quartz) are added to the vial and mixed. The mixture is centrifugated 5 min at 3,000 rpm and the supernatant withdrawn. Pellet is resuspended in 1 ml of distilled water and a small amount of an anion exchange resin (AGI-X10, 100-200 mesh, Bio Rad Lab) is added to remove the free iodide. The mixture is centrifugated again and the pellet redissolved in acetic-acetone-distilled water (1-20-79 vol). After a last centrifugation the tracer (HCT*) retained in the supernatant is diluted with labeling buffer and kept stored at —20° C in 0.5 ml aliquots until use.

*Purification.* HCT* can be used within fifteen days without purification. After this time, a chromatographic purification is needed. The chromatography is performed through a 0.9 × 60 cm Sephadex G-50 (Pharmacia Fine Chem) column, eluted with 0.02 M, pH 7.5 phosphate buffer with 0.001 % sodium EDTA and 0.01 % merthiolate. Eight hundred microliter fractions are collected. Radioactivity is measured in a Nuclear Chicago gamma counter with an approximate efficiency of 70 %.

*Immunoreactivity.* Lyophilized rabbit antiserum to HCT (Calbiochem A.G.) is resuspended in buffer, and used at a dilution which gives values between 40 and 60 % of HCT* bound in absence of cold HCT. Phosphate buffer 0.05 M, pH 7.5, containing 0.5 % BSA is used in the assay. The tracer is added after 96 hours of preincubation at 4° C, and the reaction is continued for another two days at 4° C. The separation of bound and free fractions is performed by adding 0.5 ml of a Charcoal-Dextran suspension (5%-0.5%).

*Standard curves.* Two different graphical representations for the standard curves are used, 1st: percentage $B/B_0$ *versus* cold hormone concentration is utilized. % $B/B_0 = 100\ (B\text{-}N/B_0\text{-}N)$, where B = number of cpm bound in the presence of standard or unknown unlabeled hormone, $B_0$ = number of cpm bound in the absence of cold hormone, and N = mean number of nonspecific cpm bound in the absence of antibody. 2nd: the hyperbolic equation $(y - a)(x + b) = k$, being y = cpm bound in any point, a = nonspecific cpm bound in the absence of antibody, b = position of the vertical asyntotic line to the negative abscisa (which is similar to the affinity constant Km), is employed (13). This second sys-

tem allows a better approximation to calculate very low hormone concentrations, since for $x = 0$, is $y = y_0$, and consequently: $y_0 = (K/b) + a$. The amount of cold hormone in all the samples is obtained using the equation $x = (K/y - a) - b$. Sensibility at «zero» point is then (13), $S_0 = 0.01\ b\ (1 + a\ b/K)$.

Each standard curve is provided with a Charcoal blank in triplicate.

*Samples.* Blood is drawn from the cubital vein using a hypodermic syringe. After clotting at room temperature serum is obtained by centrifugation. Samples are kept frozen at —20° C until used. Native serum in 0.1 ml aliquots is used in the assay. Each serum sample is checked in triplicate for nonspecific Charcoal absorption.

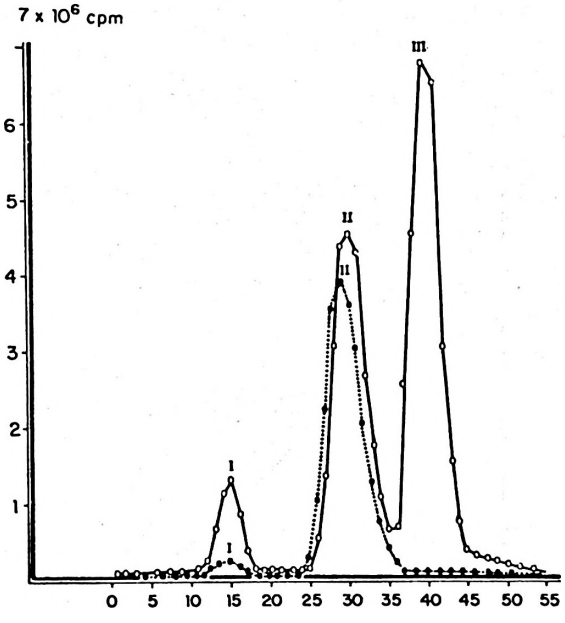


Fig. 1. *Sephadex G-50 purification of HCT* after labelling with $^{125}I$.*
Dashed line show the results with the use of Quso G-32 and the AGI-X10 resin, and continous line without any additive in the labelling procedure. (Fraction number in the abscissae.)

*Stimulation tests.* HCT secretion is stimulated by calcium infusion on pentagastrin injection. The calcium infusion test is started at 8.30 AM after an overnight fasting period. Fifteen mg/kg of calcium element was administered by iv infusion of calcium lactogluconate in 500 ml of saline over a 4-hr period. Blood samples were collected at 0, 15, 30, 60, 120, 180 and 240 min. The pentagastrin test is performed under the same conditions by rapid iv injection of 0.5 μg/kg in 5 ml of saline. Blood samples were drawn at 0, 1, 3, 5, 15, 30 and 60 min.

## Results

Labelled hormone with a mean specific activity of 380 mCi/mg (200-520 mCi/mg) and high stability («damaged» fraction and free isotope represents less than 4 % and 8 % respectively after 15 days of labelling) is obtained using the technique previously described.

Column gel filtration tests (Sephadex G-50) of HCT* aliquot shows a clear reduction of the «damaged» (peak I) and free isotope fraction (peak III) compared with the purification profiles in which neither silica nor resin are employed (figure 1). The presence or absence of BSA in the eluting buffer is not critical because similar results are obtained in both cases (fig. 2).

Significant differences between the immunoreactivity of the ascending and descending branches of the hormonal peak have not been observed.

Gastrin, pentagastrin, insulin and glucagon do not cross-react with HCT-antiserum. Specificity of HCT-antiserum is also shown by linearity of dilutions of sera with high HCT content (table I).

Dextran coated Charcoal absorbs more than 95 % of free HCT* with high reproducibility (coefficient of variation of 5 ± 2 %). Other separation methods such as Polyethylenglycol or Dioxane give worse reproducibility.

Figures 3, 4 and 5 show the results obtained submitting 10 standard curves data (mean ± S.D.) to both graphical representations described above.

Recovery of cold hormone added in five different assays is 101 ± 6 % for 1 ng/ml, and 97 ± 7 % for 0.5 ng/ml concentrations.

Determinations of HCT are systematically done in serum as no significant differences between values in serum and plasma are found. Serum kept at —20° C maintains 90 % of its original immunologic activity up to a maximal storage period of 4 months.

No significant differences in standard curves performed using incubation buffer of HCT free sera (sera of thyroidectomized patients or sera treated with Quso G-32) are obtained.

From our experimental data a sensi-

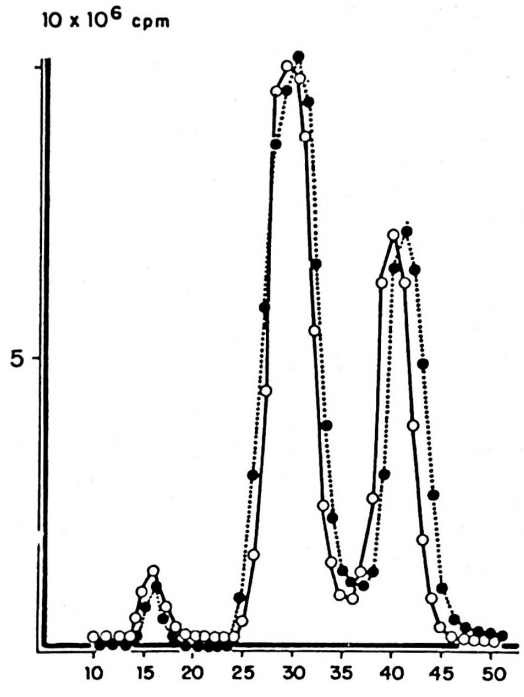


Fig. 2. *Sephadex G-100 purification of HCT* with (dashed line) or without (continous line) bovine seroalbumin in the eluting buffer.* (Fraction number in the abscissae.)

Table I. *Linearity of increasing amounts of various serum samples.*

| Native serum | 1/2 | 1/5 | 1/10 | 1/15 | 1/20 | Coefficient of correlation |
|---|---|---|---|---|---|---|
| HCT (ng/ml) | | | | | | |
| 73.0 | 32.1 | 12.0 | 6.9 | 3.5 | 2.9 | 0.998 |
| 54.1 | 29.1 | 13.0 | 5.8 | 4.5 | 3.2 | 0.998 |
| 31.0 | 14.1 | 7.1 | 4.2 | 2.9 | 1.9 | 0.997 |

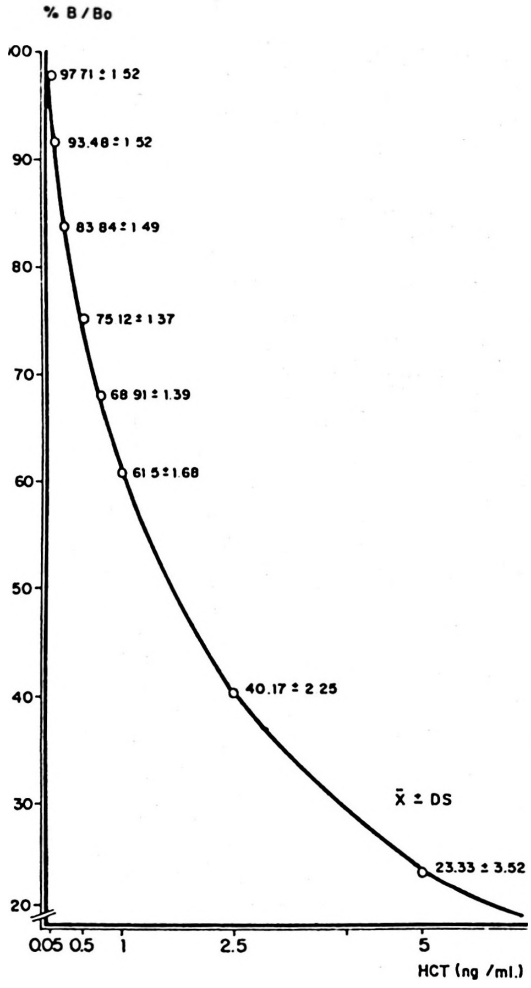


Fig. 3. *Graphic representation of the X ± S.D. of ten different standard curves in the form %B/Bo versus cold hormone concentration.*

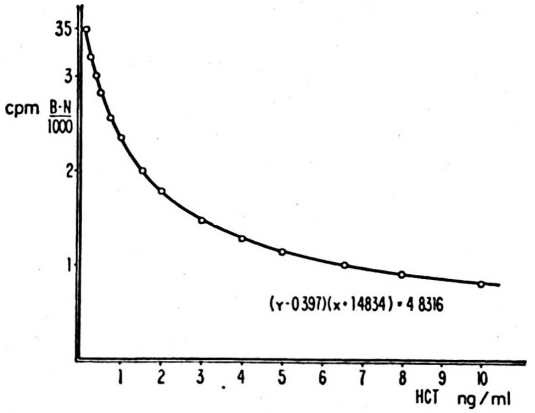


Fig. 4. *Graphic representation of the same mean standard curve in hyperbolic form:* $(y - a)(x + b) = k$.

bility, or minimum amount of detectable hormone statistically different from zero, of 60 pg/ml is obtained.

Intra-assay coefficient of variation (C.V) of ten determinations of the same serum pool is found to be 7.5 %. Inter-assay C.V amounted 17 % in ten different assays. The mean value of HCT in 110 fasting normal persons is 227 ± 123 pg/ml (undetectable 8.2 %). No significant difference is found between basal levels of HCT in men (241 ± 123 pg/ml) and women (217 ± 114 pg/ml).

No correlation between basal levels of HCT and serum ions (calcium and magnesium measured by atomic absorption spectrophotometry, sodium and potassium by flame photometry and phosphorus by the Fiske and Subbarow method) is observed.

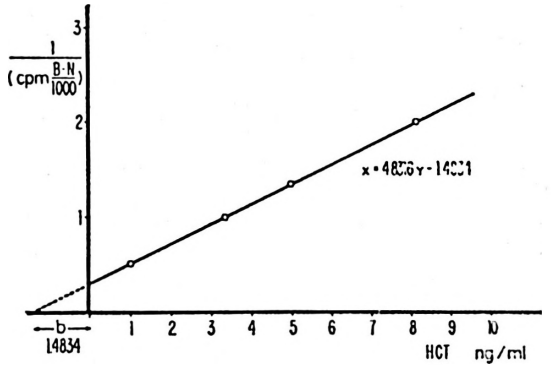


Fig. 5. *Linear transformation of the curve* $(y - 0.397)(x + 1.4834) = 4.8316$ *performed using the reciprocal method.*

In all six normal subjects significant ($p < 0.01$) increases in plasma immunoreactive HCT occurred after calcium administration. HCT reaches its maximum level between 60 min and 120 min after the beginning of the infusion, and this value never exceeding 900 pg/ml. Correlation between serum HCT and calcium is not significant ($r = 0.20$).

In seven patients with medullary thyroid carcinoma (MTC) basal HCT levels ranged between 5,000 and 110,000 pg/ml, the difference towards controls being highly significant ($p < 0.001$). In three of them it was possible to perform a calcium infusion obtaining an average 4.25 fold increase of basal HCT levels. The correlation between the raising in HCT ($p < 0.01$) and calcium levels is statistically significant ($r = 0.74$; $p < 0.01$).

Intravenous injection of pentagastrin increases very quickly and significantly ($p < 0.001$) HCT levels in normal individuals, in two patients with Paget's disease (whose basal HCT values are within the normal range), and in six patients with non medullary thyroid carcinoma whose basal HCT levels are also within the normal range. The maximal HCT response to pentagastrin occurs within 3 min after injection, and the maximum HCT values found are never higher than 1.5 ng/ml in normal persons, patients with Paget's disease and patient with non medullary thyroid carcinoma. In two patients with MTC the maximal response to pentagastrin are also within 3 min after injection, and peak values show a 7.5 and 10-fold increase, respectively.

## Discussion

Despite being a little sized peptide (about 3,500 daltons) and having only one

thyrosine residue (11), HCT can be easily lebelled with $^{125}I$ and preparations of HCT* with high specific radioactivity are obtained. After 25 days the immunoreactivity of labelled HCT is significantly reduced; hence the sensibility of standard curves also decreases.

In contrast to other authors (6), our results show that BSA saturation of the gel column or the presence of this protein in the eluting buffer, does not modify the elution volumes of the «damaged fraction», hormonal peak and free isotope (fig. 2). This may be due to the small amount of BSA (0.1 ml at 1 %) added to the labelling vial.

Dextran coated Charcoal separation method gives in our experience a better reproducibility and lower non-specific binding than Dioxane (7, 14) o Polyethylenglycol (3) precipitation.

The sequential saturation technique used in this assay provides a higher sensibility compared to the equilibrium technique but it requires a more accurate control of incubation time (17).

Specificity of the antiserum used is considered to be adequate as shown by linearity of serial dilution of sera with high HCT content (sera of MTC). Also the absence of cross reactivity with glucagon, insulin, gastrin and pentagastrin, allow us to exclude possible interferences of these hormonal peptides in our assay.

There is still disagreement about the true limits for normal serum HCT levels in man. The heterogeneity of endogenous HCT, varying affinities of different antibodies, antisera recognition of different binding sites of HCT, and nonspecific interference of the assayed sera, may explain the divergent results reported by various investigators (5, 15).

In our RIA only 8.2 % of 110 normal persons studied show undetectable HCT basal levels, and never exceeding 460 pg per mililitre.

Calcium infusion significantly increases basal levels of HCT in normal individuals and in patients with MTC. However there is no significant correlation between calcemia and HCT levels. Due to this fact it is generally accepted that HCT stimulation is more in connection with changes in calcium levels than the absolute calcium values themselves (4, 8).

In the literature calcium infusion test induces a smaller response of HCT in some cases of MTC, whereas pentagastrin stimulation always gives the great elevation typical of this tumor type (8). Our experience confirms these findings, showing the pentagastrin stimulation in MTC about twice the response of calcium infusion. This pentagastrin stimulation test appears to be useful for clinical practice because of this simplicity and safety.

*Acknowledgements*

The authors wish to thank Ciba-Geigy for his generous supply of synthetic human calcitonin, and Dr. TRESGUERRES for his excellent assistance in the preparation of this manuscript.

## Resumen

Se ha estandarizado un radioinmunoensayo para determinar los niveles séricos de calcitonina (HCT) en personas normales y en individuos afectados de diferentes patologías. El marcaje de HCT se realiza siguiendo la técnica de la Cloramina T de Hunter y Greewood. Añadiendo al vial de marcaje sucesivamente sílice en polvo (Quso G-32) y una resina de intercambio aniónico (AGI-X10) se reducen las fracciones de hormona «dañada» e isótopo libre originadas durante su conservación. Los sueros conservados a —20° C mantienen un 90 % de su inmunorreactividad original hasta un máximo de 4 meses después de su extracción. El nivel basal medio de HCT en 110 individuos utilizados como control es de 227 ± 123 pg/ml, sin diferencia significativa entre los valores hallados en hombres y mujeres, y sin correlación con diferentes iones séricos. En siete pacientes con carcinoma medular tiroideo (CMT) la cifra basal de HCT oscila entre 5 y 110 ng/ml, mientras que, en seis casos de car-

cinoma tiroideo no medular se halla dentro de límites normales. Se utilizan dos métodos para estimular la secreción de HCT: infusión prolongada de calcio e inyección i.v. de pentagastrina. La elevación en los niveles basales de HCT provocada con la pentagastrina se produce antes y es de mayor intensidad que la observada con la infusión de calcio. Sólo en pacientes con CMT la elevación en las cifras basales de HCT obtenida con la infusión cálcica guarda correlación significativa con los de la calcemia.

## References


1. DEFTOS, L. J.: *Metabolism*, **20**, 1122-1128, 1971.
2. DEFTOS, L. J., POWELL, D., PARTHEMORE, J. G. and POTTS, J. T. Jr.: *J. Clin. Invest.*, **52**, 3109-3114, 1973.
3. DESBUQUOIS, B. and AURBACH, G. D.: *J. Clin. Endocrinol.*, **33**, 732-738, 1971.
4. FRANCHIMONT, P. and HEYNEN, G.: Parathormone and Calcitonin Radioimmunoassay in various Medical and Osteoarticular Disorders. Masson Inc., París, 1976.
5. GRAY, T. K. and ONTJES, D. A.: *Clin. Orthop. Relat. Res.*, **111**, 238-256, 1975.
6. HABENER, J. F., DEFTOS, L. J. and POTTS, J. T. Jr.: *Clin. Chim. Acta*, **39**, 407-415, 1972.
7. HABENER, J. F., MAYER, G. P., POWELL, D., MURRAY, T. M. and SINGER, F. R.: *Clin. Chim. Acta*, **45**, 225-233, 1973.
8. HENNESSY, J. F., WELLS, J. A. Jr., ONTJES, D. A. and COOPER, C. W.: *J. Clin. Endocrinol. Metab.*, **39**, 487-495, 1974.
9. HUNTER, W. M. and GREENWOOD, F. C.: *Nature*, **194**, 495-496, 1962.
10. HUNTER, W. M.: In «Radioimmunoassay Methods» (K. E. Kirkham and W. M. Hunter, eds.), Churchill Livingstone. Edinburgh, 1971, pp. 3-23.
11. NEHER, R., RINIKER, B., RITTEL, W. and ZUBER, H.: *Helv. Chim. Acta*, **51**, 1900-1905, 1968.
12. SILVA, O. L., SNIDER, R. H. and BECKER, K. L.: *Clin. Chem.*, **20**, 337-339, 1974.
13. TAMARIT, J.: *XV Reunión S.E.C.F.* Mesa Redonda de Radioinmunoensayo. Zaragoza, 1975, pp. 87-98.
14. THOMAS, K. and FERIN, J.: *J. Clin. Endocrinol.*, **28**, 1667-1670, 1968.
15. TASHJIAN, A. H.: In «Methods in Investigative and Diagnostic Endocrinology» (S. A. Berson and R. S. Yalow, eds.), North Holland, Amsterdam, 1973, pp. 1010-1019.
16. WOLFE, H. J., WORLKEL, E. F. and TASHJIAN, A. H.: *J. Clin. Endocrinol. Metab.*, **38**, 688-694, 1974.
17. ZETTNER, A. and DULY, P.: *Clin. Chem.*, **20**, 5-14, 1974.