**Analytical validation of the Liaison Calcitonin_II-Gen (DiaSorin).**

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Running title: Validation of a calcitonin method: Liaison II Gen®

This paper has never been submitted before. Some parts of this work have been accepted as an abstract for presentation at the AACC 2010 (Anaheim) Congress.

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Abstract.

Background. We validated the DiaSorin Liaison Calcitonin_II-Gen, an improved method for calcitonin (CT) determination, compared this method with the Cisbio_h-CT kit and established the reference range of CT in a normal adult population.

Methods. We determined the precision, functional sensitivity, traceability to the 2nd IS 89/620, linearity and the measurement uncertainty, accuracy profile and β-expectation limits. We evaluated the specificity, the susceptibility to HAMA, hook-effect and carry-over. To establish a reference range, we selected 267 non-renal insufficient adults presenting normal TSH, free-T4 and calcium levels and no anti-thyroglobulin antibodies as our "reference" healthy population. We compared the method with Cisbio on 250 consecutive and 45 samples post-pentagastrin stimulation test.

Results. Precision (expressed as CV) was <10% for the measurement range, functional sensitivity: 5.3 ng/L and the method was found linear until the 1/10 dilution. Uncertainty ranged from 25 to 7.2% and the risk that one result falls out of the ±20% acceptance limits was <5% between 2.9 and 1513 ng/L. The Bland and Altman plot showed no systematic bias between the two methods. The test is still prone to HAMA influence, does not present any hook-effect but a carry-over was observed. Ninety-five percent of our adult reference population presented CT levels < 7.4 ng/L, with an important gender difference: 95% of the men presented CT values <9.8 ng/L whereas 95% of women were < 4.0 ng/L.

Conclusions. Liaison Calcitonin_II-Gen is an analytically robust method. The important gender-difference observed in our well-designed population might lead to a re-evaluation of the generally used "10 ng/L" cut-off in a multicentre prospective study.

Keywords: analytical validation; calcitonin; measurement uncertainty; reference range; medullary thyroid carcinoma

Introduction.

Calcitonin (CT) is a 32-amino acid calcium lowering peptide secreted by the C cells (parafollicular cells) of the thyroid. Serum CT is the most specific and sensitive marker of medullary thyroid carcinoma (MTC) for both the primary diagnosis and the post-chirurgical follow-up (1;2). Although CT levels are higher in males than in females, many laboratories use a cut-off value of 10 ng/L instead of a population-based reference interval. Indeed, different studies have shown that, in normal population and in 90% of patients suffering from other nodular thyroid diseases, basal CT concentration were below 10 ng/L (3-7). In clinical practice, patients with basal CT > 10 ng/L should undergo a pentagastrin stimulation test to exclude the presence of MTC. Stimulated peak serum CT concentration > 100 ng/L are >90% specific for the diagnosis of C-cell disease, either C-cell hyperplasia or MTC (1;6;7). It is however important to point out that most of the reference studies have been established on the basis of data obtained with the Cisbio International immunoradiometric calcitonin assay (Gif-sur-Yvette, France). Even if this kit is still recommended by some scientific societies, many laboratories have moved to more automated methods, either the Siemens Immulite 2000 family or the DiaSorin Liaison. We (8) and others (9) have already warned against the significant problems when the “10 ng/L” cut-off was indiscriminately used with the first version of the Liaison calcitonin kit.

Recently, DiaSorin (Stillwater, MN) launched a new version of the calcitonin kit (Calcitonin_II-Gen) on the Liaison platform. The aim of this study was to evaluate the analytical performance of this new version of the Liaison calcitonin and to compare the performance of this kit with the Cisbio International immunoradiometric method. We also determined the biological reference interval of this parameter in a very well biologically described “normal” adult population.

## 2. Materials and methods.

### *2.1 Analytical methods*

The Liaison Calcitonin_II-Gen assay is a one step sandwich chemiluminescent assay that uses a pair of affinity-purified mouse antibodies. The kit is calibrated against the 2nd International Standard 89/620.

The Cisbio International IRMA h-CT is a solid phase two-site immunoradiometric assay that uses a pair of monoclonal antibodies, one coated on a solid phase and the other radiolabelled with $^{125}I$. The manufacturer claims that 1µIU of the 2nd International Standard 89/620 corresponds to 3.6 pg of calcitonin.

### *2.2 Statistical softwares*

We used the Medcalc (Mariakerke, Belgium) and the e-noval (Arlenda, Liège, Belgium) softwares for the statistical evaluation of the results.

### *2.3 Samples*

We only used serum samples for CT determination. All the samples were treated according to our pre-analytical procedure: after sampling, they were spun at +4°C at 3500G, aliquoted and kept frozen at -20°C until determination.

### *2.4 Validation protocol*

For the validation purpose, we evaluated the precision in accordance with a modified protocol based on CLSI EP-5A2: 12 serum pools were assayed in six replicates per day on five different days (360 determinations). The limit of quantification (or functional sensitivity) was assessed as the lowest values giving in inter-assay (on 10 different days) a coefficient of variation of 20%. The linearity was evaluated based on CLSI EP-6A. We studied the traceability to the 2nd International Standard 89/620. We evaluated the measurement uncertainty, the accuracy and the β-expectation limits: measurement uncertainty characterizes the dispersion of the values around the true value and β-expectation tolerance limits with

β=0.95 are the upper and lower values in-between which each future measurements of the same level has a probability of 95% to be found (10;11). We consider that the method will provide accurate results if the 95% β-expectation tolerance interval at each concentration level is fully included in the acceptance limits that we decided to settle at ±20% (12).

We checked the specificity of the assay by using 14 samples from patients treated by complete thyroidectomy and irradiation.

The presence of a hook effect was studied with samples spiked with very high amounts of the 2nd IS 89/620 and, finally, we studied the susceptibility of the kit to present an interference with human anti-animal antibodies (HAMA) with 3 selected samples, known to present an interference with the previous version.

*2.5 Comparison with the Cisbio h-CT*.

We compared the results obtained with the two methods in 250 consecutive samples from our daily routine and 45 samples obtained after pentagastrin stimulation test.

*2.6 Establishment of the reference range*.

We evaluated the biological reference interval in a healthy adult population (122 women and 145 men; mean age: 56.4 yo (L:17; H: 87)), selected on basis of the absence of anti-thyroglobulin antibodies and normal levels of TSH (0.3 – 3.6 mIU/L) and free T4 (10.2 – 21.8 pmol/L) on Liaison, according the manufacturer. All the patients presented normal levels of serum calcium (2.20 – 2.60 mmol/L) and did not suffer from chronic kidney disease (estimated glomerular filtration rate > 60 ml/min/1.73m² according to the MDRD equation). We used the Kolmogornov-Smirnov test to check if the distribution of the parameter in the population was Gaussian. If it wasn't the case, we used a non-parametric method to calculate the reference range according to the CLSI C28-A3 guideline (13). We took the 95% right-sided result as the higher limit of normality.

## 3. Results.

### *3.1 Values observed in the population*.

Our selected population for the establishment of the biological reference interval in adults presented a median TSH level of 1.35 mIU/L (Lowest: 0.366; Highest: 3.30) and a mean free T4 of 14.6 pmol/L (L:10.2 – H: 21.1). The distribution of calcitonin in the population was not normally distributed (p-value <0.0001) and we thus used the non parametric method to evaluate the reference range. The calcitonin levels observed in men were significantly higher than those obtained in women (median: 3.0 vs. 0.6 ng/L, respectively; p-value <0.0001). Ninety-five percent of the male population presented CT levels < 9.8 ng/L (90% CI: 7.4 – 12.9 ng/L) whereas 95% of women were below 4.0 ng/L (90% CI: 3.2 – 5.16 ng/L). Taken as a whole, 95% of the reference population presented calcitonin levels below 7.4 ng/L and 97.5% were <11.3 ng/L. The distribution of the calcitonin levels in our reference population is shown on Figure 1.

### *3.2 Analytical performance*.

The results of the precision evaluation are shown in Table 1. As can be seen from this Table, repeatability did not exceed 10% and the intermediate precision 11% in the concentration range 2.9 to 1503 ng/L. The functional sensitivity was established at 5.3 ng/L. The kit was correctly traceable against the 2$^{nd}$ IS 89/620, as 1µIU corresponded to 5.5 pg of Liaison calcitonin (expected: 4.8 – 5.7 pg). Mean recovery was 98.8 ± 4.2 %. The method was found to be linear until the 1/10 dilution. Measurement uncertainty and β-expectation tolerance limits observed on the 12 pools studied is presented in Table 2. Measurement uncertainty was comprised between 25.0 and 7.2%.

The β-expectation tolerance intervals were computed at each concentration level with a probability β=95%. This means that, on average, 95% of the future results that will be generated by this method will be included in the computed tolerance intervals. Indeed,

tolerance interval methodology is predictive. The method will provide accurate results if the 95% β-expectation tolerance interval at each concentration level is fully included in the acceptance limits that were settled at ±20%. Figure 2 illustrates this through an accuracy profile. As shown on this Figure, the method provided accurate results from 5.3 to 1513 ng/L. These two concentration values are the lower and upper quantitation limits, respectively. They thus define the dosing range of the method. Indeed, it is the guaranteed that each future result will be included in the ±20% with a probability of 95%.

Among the 14 samples from patients treated by complete thyroidectomy and irradiation, 13 presented undetectable (<1 ng/L) levels of CT, one only presenting a value of 1.15 ng/L.

We did not observe any hook effect with samples presenting values up to 1 million ng/L, but there was a carry-over, with a blank sample presenting a value of 8 ng/L after being assayed after the 1 million ng/L sample.

We had selected 3 samples that presented spurious elevated values with the first version of the kit (17, 43 and 91 ng/L) due to HAMA interference. Indeed, after treatment with the Scantibodies heterophile blocking tubes (HBT: Scantibodies, Santee, CA), their levels returned to <10 ng/L and when determined with the Cisbio kit, these three samples presented a value of respectively 9.3, 2.7 and 0.1 ng/L. In these 3 samples, the Liaison Calcitonin II Gen gave also spurious results (10.1, 71 and 113 ng/L), that returned below 5 ng/L after HBT treatment.

*3.3 Comparison with Cisbio h-CT*

We compared the two methods on 250 consecutive patients with the Bland and Altman plot (Figure 3). As can be seen on this figure, there was no systematic bias between the two methods (mean difference = 0.1 ng/L) and the standard deviation of the differences was found to be 2.0 ng/L. We did not find any significant difference with the Wilcoxon test (Median (95% IC): Liaison: 1.62 ng/L (1.32 – 2.03) and Cisbio: 1.53 ng/L (1.31 – 2.0)). In the 45

samples obtained after pentagastrin stimulation (range: 9.3 – 838 ng/L), the Bland-Altman plot showed a mean difference of 11.1±49.3 ng/L. No statistical difference was observed between the two methods.

## 4. Discussion.

We presented here the results of the analytical validation of the DiaSorin Liaison Calcitonin_II-Gen assay. Our results show that this method is sensitive and precise. We also established the measurement uncertainty and we have shown that between 5.3 and 1513 ng/L, 95% of the results will be in the ±20% maximum allowable total error. The results of the Liaison Calcitonin_II-Gen are in accordance with those obtained with the Cisbio h-CT, which is the method that had been used to develop the different interpretative guidelines. The Calcitonin_II-Gen is correctly calibrated against the 2$^{nd}$ IS 89/620. However, this new version of the kit is still sensible to HAMA interference: samples presenting values higher than 10 ng/L should be treated to remove this interference.

In 2007, Bieglmayer et al. had checked the specificity of the first version of the Liaison CT kit by using 15 samples from patients treated by complete thyroidectomy and found detectable calcitonin levels in 9 of them. They concluded that this former assay was prone to non-specific effects causing incorrect CT detection in patients free of thyroid tissue (14). We found here that 1 among 14 thyroidectomized patients presented detectable CT levels. This patient had actually recently been diagnosed as suffering from a seronegative form of polyarthritis. Our tests for RF or HAMA interference are inconclusive at such a low CT level and we could not thus conclude if we observed an analytical interference or a lack of specificity of the assay for this particular patient. Nevertheless, we can conclude that the specificity of the new Liaison CT assay is greatly improved compared to the former one.

According to d'Herbomez *et al*. (15), we defined our "normal" population as a population of adult men and women presenting normal levels of TSH, free T4, and calcium levels. These patients did not present anti-thyroglobulin antibodies and did not suffer from renal insufficiency. Unfortunately, we had no information on the smoking status of these patients. As expected, the results observed were much lower in women, compared to those observed in men. Even if 95% of our "normal" patients presented CT levels below 7.4 ng/L – and thus below 10 ng/L – the 95$^{th}$ percentile of the women population was very low, at 4.0 ng/L. The Liaison Calcitonin_II Gen is thus still lacking sensitivity, as more than 95% of the values observed in women will be responded as "<5.3 ng/L".

Machens et al. have recently claimed for gender-specific CT thresholds to screen for occult MCT (16). On the other hand, Rink *et al*. have advocated that a cut-off of 15 ng/L (as established with the IBL Calcitonin IRMA or Medipan Calcitonin-IRMA magnum) instead of the "traditional" 10 ng/L for basal CT was able to detect all MTC and reduced false-positive cases (17). The debate on the use of a "clinical" cut-off value or cut-off values derived from apparently healthy people for MTC screening is thus still ongoing. Our study was purely analytical and neither designed nor powered to establish CT "clinical" cut-offs. However, in the light of our results, it could be interesting to re-evaluate the "10 ng/L "cut-off in a large multicentre prospective study, across a wide spectrum of well-validated CT assays. This point is particularly important as the transposition of clinical cut-offs established with one method to other methods have shown important limits (see PTH cut-offs in the former KDOQI Guidelines or GH cut-offs in the diagnosis of growth-hormone deficiency).

Finally, whatever the cut-off value(s) to be used, one should not forget that basal serum CT concentrations should always be interpreted according to the clinical context. Indeed, it presents a low predictive positive value of 15.4%, reflecting many false-positive results, potentially leading to unnecessary surgery (18). Nevertheless, CT measurement is still

recommended by the European Thyroid Cancer Taskforce in the initial diagnostic evaluation of thyroid nodules (19) and a recent American study concluded that routine CT screening for MTC in the patients with thyroid nodules could be as efficient as widely accepted screening programs, like measurement of TSH, colonoscopy and mammography screening (20).

Conflicts of interest: none to declare.

1 Table 1: Precision observed on twelve serum pools.

| Pool | n | Mean | Within-run | | Total | |
|---|---|---|---|---|---|---|
| | | (ng/L) | SD | CV(%) | SD | CV(%) |
| 1 | 30 | 0.98 | 0.27 | 27.6 | 0.29 | 29.6 |
| 2 | 30 | 1.46 | 0.19 | 13.0 | 0.21 | 14.4 |
| 3 | 30 | 2.94 | 0.27 | 9.2 | 0.35 | 11.9 |
| 4 | 30 | 6.10 | 0.51 | 8.4 | 0.51 | 8.4 |
| 5 | 30 | 7.28 | 0.42 | 5.8 | 0.52 | 7.1 |
| 6 | 30 | 14.1 | 0.8 | 5.7 | 0.9 | 6.4 |
| 7 | 30 | 15.3 | 1.0 | 6.5 | 1.0 | 6.5 |
| 8 | 30 | 23.0 | 1.0 | 4.3 | 1.0 | 4.3 |
| 9 | 30 | 71.0 | 3.4 | 4.8 | 3.4 | 4.8 |
| 10 | 30 | 219 | 9.2 | 4.2 | 10.5 | 4.8 |
| 11 | 30 | 1159 | 10.5 | 0.9 | 34.4 | 3.0 |
| 12 | 30 | 1503 | 18.9 | 1.3 | 50.0 | 3.3 |

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Table 2: Measurement uncertainty and β-expectation tolerance limits observed on 12 pool samples.

| Pool | Mean | Uncertainty | Relative uncertainty | β-expectation tolerance limit | Relative β-expectation tolerance limit |
|---|---|---|---|---|---|
| | (ng/L) | (ng/L) | (%) | (ng/L) | (%) |
| 1 | 0.98 | 0.30 | 60.9 | [ 0.37 , 1.60] | [-62.3 , 62.3] |
| 2 | 1.46 | 0.22 | 30.2 | [ 1.00 , 1.92] | [-31.2 , 31.2] |
| 3 | 2.94 | 0.37 | 25.0 | [ 2.15 , 3.72] | [-27.0 , 27.0] |
| 4 | 6.10 | 0.52 | 17.1 | [ 5.03 , 7.17] | [-17.5 , 17.5] |
| 5 | 7.28 | 0.55 | 15.1 | [ 6.12 , 8.44] | [-16.0 , 16.1] |
| 6 | 14.1 | 0.9 | 12.9 | [ 12.2 , 16.1] | [-13.7 , 13.6] |
| 7 | 15.3 | 1.0 | 12.8 | [ 13.3 , 17.3] | [-13.1 , 13.1] |
| 8 | 23.0 | 1.0 | 9.0 | [ 20.9 , 25.2] | [-9.5 , 9.5] |
| 9 | 71.0 | 3.5 | 9.8 | [ 63.7 , 78.2] | [-10.3 , 10.3] |
| 10 | 219 | 11.0 | 10.0 | [ 196 , 243] | [-10.7 , 10.7] |
| 11 | 1159 | 37.5 | 6.5 | [ 1060 , 1258] | [-8.5 , 8.5] |
| 12 | 1503 | 54.3 | 7.2 | [ 1363 , 1642] | [-9.3 , 9.3] |

Figure 1: Box-and-whisper plot of the calcitonin values observed in our reference population (267 Individuals) with the Liaison Calcitonin_II-Gen. The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The “10 ng/L” cut-off is highlighted by a solid line

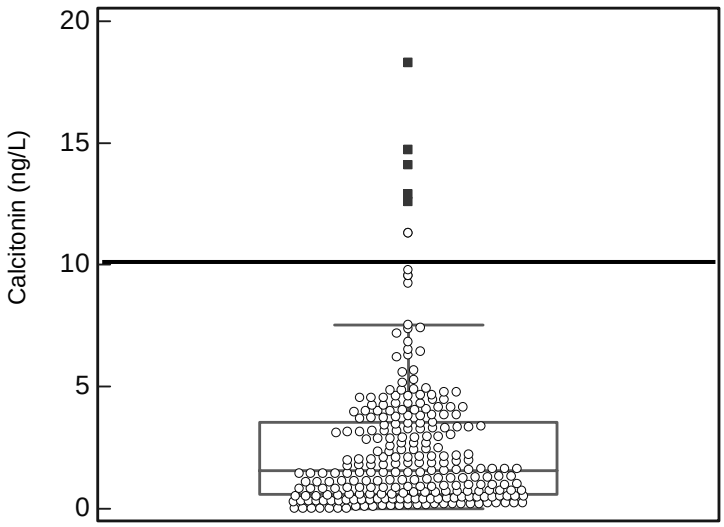

Figure 2: Accuracy profile of the Liaison Calcitonin_II-Gen assay. When the β-expectation limits (broken lines) are comprise between the maximum total allowable error (settled here at ±20%), the method is considered as valid.

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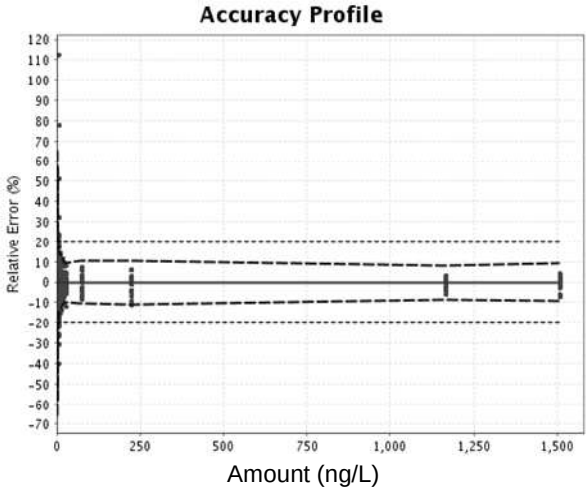
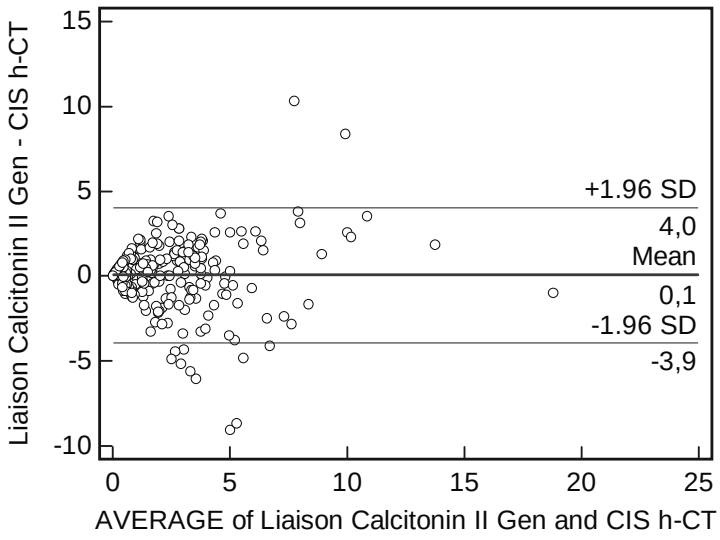


Figure 3: Bland and Altman plot for calcitonin results obtained in 250 consecutive patients with the Liaison Calcitonin II Gen and the Cisbio h-CT. Results are given in ng/L.



Legend of the Figures:

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