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Statements and Declarations

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Véronique Raverot and Pauline Perrin. The first draft of the manuscript was written by Véronique Raverot and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Abstract (200 words) (150 to 250 words)

Purpose: Measurement of prolactin in clinical laboratories is an important part of the management of patients with pituitary adenoma. Prolactin is known to be sensitive to the high-dose hook effect, in the presence of extremely high concentrations. This interference is mentioned in most recent articles discussing prolactin assay and management of pituitary prolactin adenomas [1-3]. The objective of our study was to evaluate if the mention of the high dose hook effect is relevant in actual practice.

Methods: A serum from a patient with giant macroprolactinoma was assayed by using all the different reagents available in France in 2020 on native serum and after dilution. Technical inserts from manufacturers were reviewed to study information of analytical principles, numbers of steps, and mention to high dose hook effect if necessary.

Results: Fourteen reagents were studied; all were two-site immunometric assays, mostly in 1 step (11/14). One tested reagent was sensitive to high dose hook effect leading to falsely low prolactin concentration when measured on native serum.

Conclusion: The high-dose hook effect still exists. The evolution of reagents may lead to new reagents sensitive to this effect in the future. We therefore advise that this hook effect be mentioned in the recommendations.

Introduction

Measurement of prolactin in clinical laboratories is an important part of the management of patients with pituitary adenoma. Prolactin is known to be sensitive to various analytical interferences, including the high-dose hook effect, like other biochemical markers known to undergo large amplitudes of concentration variation. In the presence of extremely high concentrations of prolactin, the reagent antibodies can become saturated and fail to form a sandwich, leading to a lower than expected prolactin result [4]. This uncommon analytical pitfall, which did not exist in older competitive assays, has been described in sandwich assays, also called two-site immunometric assays when handled in one step [5,6]. This known phenomenon can be avoided by using sandwich assays that run in two steps (including a wash step before the addition of the second antibody), or by diluting the samples when using sandwich assays that run in one step [3,4].

The high-dose hook effect is mentioned in most recent articles discussing prolactin assay and management of pituitary prolactin adenomas [1-3]. However, articles describing case reports or series of high-dose hook effects in prolactin assay are mostly old or poorly documented [1,7-13]. Although few changes in the principles of immunoassays have occurred since these cases, most of the reagents have evolved. The objective of our study was to evaluate if the mention of the high dose hook effect is relevant in actual practice. We therefore propose to describe the different reagents currently available for the measurement of prolactin, to study the manufacturers' recommendations concerning the high-dose hook effect and to test these reagents in order to establish whether or not this high-dose hook effect in the measurement of prolactin still exists.

Methods

Reagents and protocol: Selection of French laboratories representative of the different techniques listed on the Probioqual (a French association for the promotion of quality control in medical biology) survey report of December 2020 was made thanks to the help of the specialized biochemistry group of the French society of nuclear medicine and Probioqual in order to be exhaustive of prolactin reagents used in France in 2020. These different laboratories agreed to provide us with the technical data sheets of the suppliers and to perform prolactin measurement on sample that we dispatched, on native serum firstly and after dilution 1:10 or more, as practiced in the laboratory when necessary.

Blood sample preparation: A single patient diagnosed with a giant macroprolactinoma was informed and agreed to participate to make this study possible. Serum samples were aliquoted and stored at -20°C until analysis. The volume of sample provided to participating laboratories was large enough (500 µL) to allow measurement on native serum and after dilution on all tested systems.

Data collected: Prolactin values obtained on native serum and after dilution were collected. The technical data sheets were reviewed to study informations of analytical principles: competitive or two-site immunometric assays; number of steps for the two-site immunometric assays : one step or two steps (with a wash step) ; linearity range; mention to high dose hook effect if necessary; and concentration until which the high dose hook effect is not supposed to occur.

Results

In the survey of December 2020, Probioqual received 464 results for prolactin measurement. Reagents were from 11 manufacturers. Two manufacturers used the same reagent on different analyzers: Architect and Alinity from Abbott were combined in one analyzers and the same for Cobas e601 and e801 from Roche. Samples with elevated prolactin concentration have been sent to 16 French laboratories to test the 14 reagents (ie also to test different analyzers from Abbott and Roche). Probioqual combined answers from those different analyzers using the same reagent (cf figure 1 and table1) and obtained 14 different analytical systems.

The careful analysis of the insert technical notice allowed an up to date evaluation of the prolactin reagent situation in 2020's:

- All reagents used two-site immunometric assays with different labels (radioactivity, chemilumininescent, electrochemiluminescent…).
- The reactions are in 1 (78.6% = 11 reagents/14) or 2 (21.4% = 3 reagents/14) steps.
- The linearity range vary from 190 to 470 µg/L depending the manufacturer.
- All the one step reagents mentioned the high dose hook effect in their notice (100%).
- The theorical concentration until high-dose hook-effect is not observed vary from 9520 µg/L to 50 000 µg/L depending the manufacturer.

Prolactin measurement: When perform on native serum, one reagent was sensitive to high dose hook effect and gave a result of 150 µg/L below the linearity range of the assay and all

the other were reported as “above the upper limit of the assay”. Results obtained after dilution varied from 17900 µg/L to 86900 µg/L depending on the reagent used (table 1). The reagent sensitive to high dose hook effect gave result of 17900 µg/L after dilution compared to 150 µg/L on native serum. This reagent is a two-site radio-immunometric assay in one step from Beckman used in France by 2 laboratories (2 results out of 464 in the Probioqual’s survey).

Except value obtained with Vista reagent, all the results obtained after dilution were above the prolactin value announced by the manufacturer as not sensitive to high dose hook effect. These results validated the absence of a high dose hook effect with the different reagents. Result obtained with Vista from Siemens led to 30800 µg/L and the limit not sensitive to hook effect was announced at 50000 µg/L.

Discussion

To our knowledge, this is the first study to determine whether the high-dose hook effect still exists with current reagents for prolactin measurement. In guidelines dealing with the management of prolactin adenomas, this analytical interference is always mentioned because it can sometimes lead to a wrong diagnosis and subsequently to wrong treatment [1], based on old publications.

A review of actual manufacturers' package inserts showed that the methodologies are based on two-site immunometric assays, which are suitable for prolactin determination. These assays, born with the discovery of monoclonal antibodies, are more sensitive and specific than previous competitive assays, but are subject to interferences that did not exist with competitive techniques, high dose hook effect for instance. This interference can be avoided by using sandwich assays that run in two steps or by diluting the samples when using sandwich assays that run in one step [3,4]. Dilution of suspected elevated concentration samples is not easy to handle in routine conditions. Interactions between clinicians and laboratories are very important; clinicians must be aware that prolactin result might be affected by an analytical interference. Some manufacturers developed some two-site immunometric assays in one step with no wash step, called homogenous phase (for example TRACE® (Time-Resolved Amplified Cryptate Emission). These technologies are supposed not being sensitive to high dose hook effect since the signal is measured several time during the reaction, but the supplier still mention in the insert that the hook effect is not observed until a notified concentration

Our study is the first one, which tested all the current available reagent. It is reassuring to observe that no hook effect occurred with modern reagent. The one from Beckman which was sensitive to high dose hook effect is an old immunoradiometric assay (IRMA) which should not be use anymore, since IRMA do not improve prolactin measurement performances.

Several case reports mentioned IRMA reagent sensitive to high dose hook effect [7,10,13,14]. We observed in this study that not all the IRMA reagent are sensitive to hook effect since that among reagents which were tested in this study, 2 were based on IRMA methodology and the one from Diasource was not sensitive to hook effect on the sample tested. Many other cases reports were seen with chemiluminescent technologies [9,11,12].

The case reports published in the literature are difficult to use: the vast majority do not mention the reagent used [8,9,12], and many cases are old with reagents that are not or no longer available. They reported that high dose hook effect is more frequent in male, which is due to the larger size of the adenoma at the time of diagnosis, and larger amount of prolactin. In future publication describing high dose hook effect or other interferences, the mention of the reagent used should be mandatory to allow a better understanding of the real systems.

This study allowed us to verify that this interference did not occur with reagents running with two steps thanks to a wash step before the addition of the second antibody [13]. Although it is theorically the easiest way to avoid the hook effect [3,4], two steps reaction is used in less than 25% of the answers collected by Probioqual in France. One major inconvenient in immunoanalysis is that reagent and analyzer are combined and it is not possible to use a 2 steps reagent if the analyzer is not adapted to it.

A major strength of this study is that the concentration of the studied sample was higher than the concentration mentioned by the manufacturers as a limit until there was no hook effect; except with one reagent (Vista Siemens). This validated the study design which was to exceed the concentration mentioned by the manufacturers. One strength of this work is that all the data sheets could be modified to take into account this new prolactin threshold; except the Vista's one.

A limitation of our work is that Probioqual's data reflect reagents used in France at the time of the survey but not the proportion of data obtained with all these reagents in clinical activities. Laboratories working with clinicians from pituitary centers may have selected reagent not sensitive to high dose hook effect. In addition, this situation is actual and we cannot preclude

the future, no one knows what future reagent will appear and one should continue to check for this interference [5].

An unexpected finding of the study was the diversity of the results obtained on the same sample: concentrations as different as 17900 to 86880 µg/L were measured on a unique sample despite adoption of World Health Organization's third international standard (IS) for prolactin 84/500, recently replaced with the 4th IS 83/573 [15]. This lack of commutability between method has already been reported [2]. This confirms the need to follow the prolactin decrement during a medical treatment with the same reagent. In 2020's, standardization of prolactin measurement needs to be done and follow by manufacturers.

Conclusion

Although extremely rare, the high dose hook effect should be mentioned in prolactin measurement in order to prevent it from being forgotten. If it is no longer mentioned, it will no longer be taught. With the multiplication of biochemistry technical platforms, the use of new reagents (not tested here) and untrained biologists/clinicians may lead to situations where the management of the patient would be altered because of this high dose hook effect.

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Manufacturers	Analyzers or reagents	Distribution of uses (%) Probioqual n=454	linearity range (µg/L)	One or two steps methods?	Concentration not sensitive to hook effect (µg/L)	Results obtained on native sample (µg/L)	Results obtained on diluted samples (µg/L)
ABBOTT	Architect / Alinity	23.4	200	2	/	>200	45946 / 45481
BECKMAN	DxI	11.9	200	1	30000	>208	51010
BECKMAN	**"IRMA"**	**0.4**	**190**	**1**	**15000**	**150**	**17900**
BIOMERIEUX	Vidas/MiniVidas	5.3	200	1	20 000	>200	86 880
DIASORIN	Liaison	0.9	377	1	33 018	>377.4	41179
DIASource	"IRMA"	0.4	202	1	18 000	>202	27819,8
Fujirebio	Lumipulse	0.2	400	2	/	>400	55294,3
ORTHO CLINICAL	Vitros	2.2	329	1	20680	>329	36317
ROCHE	Cobas e 601 / 801	37.8	470	1	12690	>470	53330
SIEMENS	Advia Centaur	6.2	200	1	30 000	>200	36592
SIEMENS	Atellica	5.9	200	1	30 000	>200	37806
SIEMENS	Dimension Vista	2.9	250	1 (LOCI)	50 000	>250	30800
THERMO FISHER	Kryptor	1.1	219	1 (TRACE)	9524	>219	37800
TOSOH	AIA	1.3	200	1	20000	>200	38295

Table 1 : Details of the technical inserts of the reagents used ; in bold the reagent sensitive to hook effect at the concentration tested.

Figure 1 : Proportion of users of the different techniques (results extracted from Probioqual data). Framed names, techniques in 2 real steps; underlined name, technique TRACE