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Médecine Nucléaire 39 (2015) 71–77
Médecine Nucléaire
Imagerie Fonctionnelle et Métabolique

General review

Pitfalls and problems in immunoanalysis

Pièges et problèmes en immunoanalyse

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Received 12 January 2015; accepted 2 February 2015
Available online 6 March 2015

Abstract

The quality and reliability of laboratory test results, and more specifically immunoassay results, depend not only on analytical accuracy and precision, but also on technical skill and knowledge of potential pitfalls and problems. It must be recalled that despite their widespread use, immunoassays can, in some circumstances, be defective. Pitfalls must be avoided from the pre-analytical phase onwards. At the analytical level, problems related to the Hoock and matrix effects as well as standardisation and interference problems that may affect all immunoassays will be discussed.

Keywords: Analytical interference; Heterophilic antibodies; HAMA; Hoock effect

Résumé

La qualité et la fiabilité des résultats d'analyses biologiques et plus particulièrement des immunodosages dépendent certes des performances analytiques des techniques utilisées, mais restent indissociables de la maîtrise ou du moins de la connaissance des pièges et problèmes susceptibles d'être rencontrés au cours de l'analyse. La banalisation des immunodosages ne doit pas faire oublier qu'ils peuvent être pris en défaut dans certaines circonstances particulières. Les pièges sont à éviter dès la phase pré-analytique. Au niveau analytique, nous aborderons les pièges liés à l'effet crochet, l'effet matrice, les problèmes particuliers de standardisation et les interférences auxquelles peuvent être sensibles tous les immunodosages.

Mots clés : Interférence analytique ; Ac hétérophiles ; HAMA ; Effet crochet

1. The pre-analytical phase

Test conditions must be carefully considered and monitored at each step of an immunoassay, from sample collection to detection of the signal used to calculate the concentration of the analyte under study. The pre-analytical phase is too often neglected but plays a vital role in quality control, as shown by the distribution of laboratory errors: 62% for the pre-analytical phase, 15% for the analytical phase and 23% for the post-analytical phase [1].

For each parameter to be measured, the biologist must provide those taking the samples with sufficient information to ensure good conditions for sampling and delivery to the laboratory. The suitability of the anticoagulant substances required for analysis must be checked whenever the analytical technique is changed. For any given parameter, the type of collection will vary depending on the assay techniques (e.g. serum, with or without separating gel, heparinated plasma, etc). Certain parameters vary with the circadian rhythm (e.g. cortisol), implying a record of the time of sampling for clinical

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http://dx.doi.org/10.1016/j.mednuc.2015.02.001
0928-1258/

interpretation. For certain hormones (e.g. ACTH and PTH), thermolability raises the risk of falsely low results so that the continuity of the cold chain must be controlled from sampling to analysis. The thyroid stimulating activity of hCG causes a decrease in TSH values in the 1st trimester of pregnancy, and of FT3 and FT4 in the 2nd and 3rd trimesters. A note of this particular physiological state or reference values appropriate for this condition should appear in the report. These are but a few examples demonstrating the key role of the pre-analytical step.

2. Basic principles of immunoassay

Immunoassay is based on the presence of immune complexes formed during the antigen (Ag) – antibody (Ab) reaction resulting from the combination of an epitope with an Ag directed against this epitope. This physiological reaction has been exploited to achieve in vitro immunoassays. The principle of immunoassay was discovered in 1953, on the occasion of the detection of molecules that interfere with insulin in the blood of diabetic patients. These substances were found to be anti-insulin Abs that patients produced when they were treated with the porcine insulin used at that time. These Abs are thus human Abs directed against an animal protein produced after allogenic immunization. Rosalyn Yalow received the Nobel Prize in 1973 for having the idea of using these specific Ac as assay reagents for assaying molecules produced in animals.

In immunoassays, the molecules being assayed are Ags. The prerequisite is thus Abs specifically directed against the Ags to be assayed. These Ags are usually macromolecules exhibiting many epitopes. They are composed of a mosaic of different antigenic determinants of which only one or two will be used in the immunoassay reaction. From a technical point of view, the Ag concentration is determined by means of a calibration curve by comparing the signal (radioactive decay, absorbance, luminescence, etc.) obtained from the sample to be assayed with the signals from standard solutions or calibration solutions of known concentrations (Fig. 1).

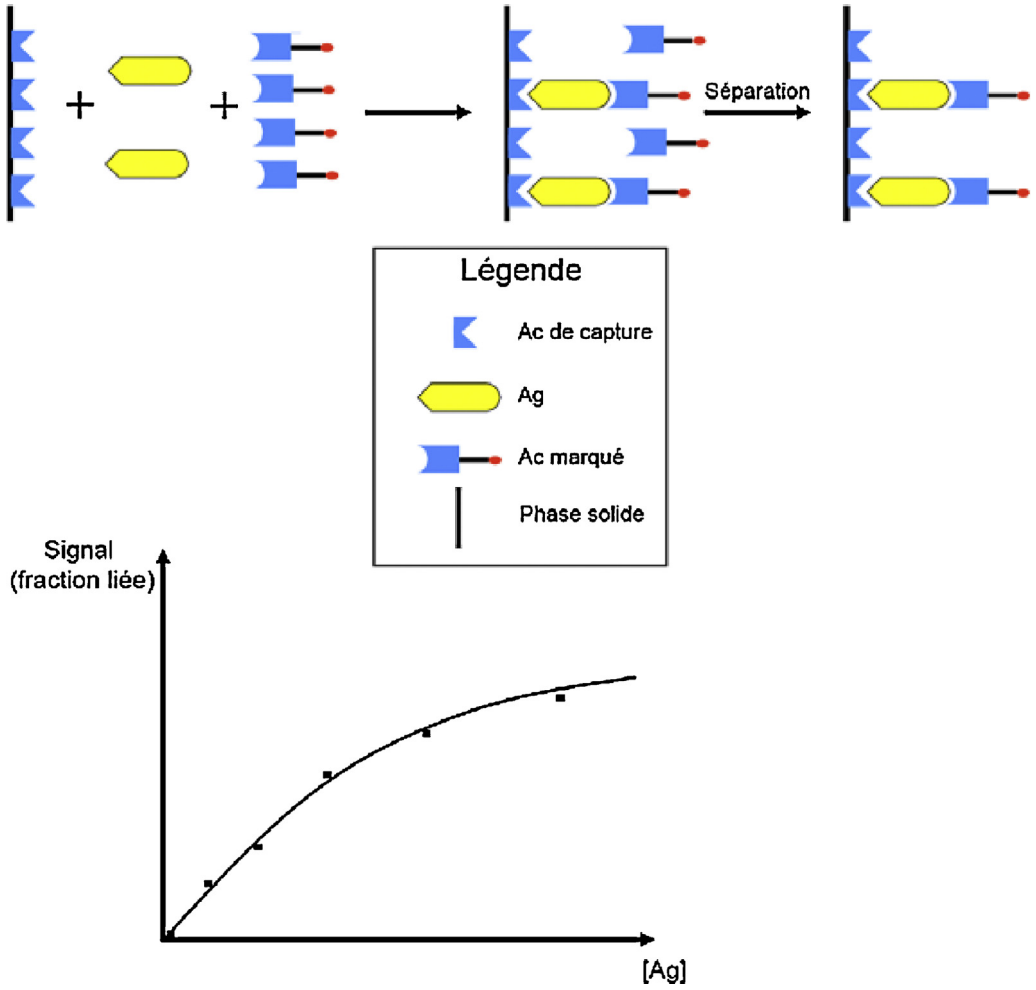

Fig. 1. Two-site immunometric method or "sandwich" technique and a schematic representation of a calibration curve.
Méthode immunométrique à 2 sites ou technique « sandwich » et sa représentation schématique d'une courbe d'étalonnage.
"[Immunoanalysis, from theory to selection criteria in clinical biology]" Chap. 2 Principles and techniques of immunoassay. Charrié A, Chikh K, Alcaraz-Galvain D. EDP Sciences 2009.

3. The analytical phase

Various problems can arise in the analytical phase itself, in particular problems related to standardisation, the Hoock effect, matrix effects or overt interference due to a lack of Ab specificity, contamination, the influence of transport proteins, or the presence of cross-reacting Abs.

The Ag is a biological entity that is represented either by a reference solution or by an International Biological Standard. For biological substances whose structure is not fully defined by chemical or physical methods, it is necessary to refer to an international standard (IS). The International Standard (IS), a preparation derived from a large collaborative multiple laboratory study using different assays, is distinguished from the International Reference Preparation (IRP) coming from a smaller study and specifically intended for immunoassay. The International Unit (IU) is defined as the biological activity contained in a defined mass of IS or IRP. When there is no IS (as is currently the case for the majority of tumour markers), the analyte concentrations are expressed in arbitrary units. Due to standardisation difficulties, it is not uncommon for different immunoassay kits to give different results on the same sample. Pure standards are easily obtained for haptens; chromatography coupled with mass spectrometry associated with isotopic dilution is a reference method. Problems related to Ab specificity, interference by transport proteins or matrix effects persist. For protein assays, standardisation is more difficult as ideally they require a match between the reference and the analyte to be determined [2,3]. These assays are sensitive to analyte heterogeneity in biological fluids (precursors, degradation fragments, complexes, heterogeneous glycoproteins, etc.) (Table 1).

3.1. The Hoock effect

The Hoock effect is found when Ab concentration is very high (Fig. 2), and occurs especially in progressive tumour pathologies (prolactinoma, or growth hormone adrenocorticotropic adenoma, insulinoma, hydatidiform moles, pancreatic cancer or thyroid carcinoma, to name but a few clinical situations). In this case, the Ag is in excess relative to the Ab present in the reaction mix and all the Ag cannot be sandwiched, so that the final Ag concentration is erroneous [4].

The industry increasingly protects its systems from this artefact by making kinetic measurements of the formation of the Ab–Ag–Ab complex, limiting the detection range and recommending the systematic dilution of potentially high concentration samples. However, this snare cannot be systematically removed. If the level of the analyte is not in accordance with the extent of metastatic spread, the sample must be diluted, which has the effect of reducing the concentration of Ag. In this case, Abs are again in excess and the response becomes linear with respect to concentration.

3.2. The matrix effect

The matrix effect is often present as the Ag–Ab reaction is largely influenced by pH, ionic strength and protein concentration. These parameters vary depending on the organic fluid in which the reaction takes place. In addition, there is often a significant difference between the chemical composition of the standard solutions used for assay calibration and that of the biological medium containing the samples to be assayed. What can be done to reduce the matrix effect? Ideally the method should be validated in the same biological medium as that of the sample to be evaluated. If this is not possible, the assay solution can be overloaded with bovine serum albumin to approximate protein concentrations in serum [5]. The ISO directive 15189 regulating medical analysis laboratories only advocates the first solution, i.e. validation of the method in the same biological fluid as that of the sample, combined with the introduction of external quality controls made in the same biological medium as the assay.

Table 1
Examples of standardisation problems and proposed solutions.
Problèmes de standardisations et solutions proposées : exemples.

Analyte	Origines des difficultés de standardisation	Matériel de référence proposé et recommandations
hCG	Hétérogénéité des formes circulantes	33° SI
PSA	Hétérogénéité des formes circulantes et raccord métrologique variable sur le standard international	PSA total : WHO 96/670 PSA libre : WHO 96/668
Thycoglobuline	Nombreuses isoformes plus ou moins iodées	Étalon commun CRM 457
GH	GH 20 kDa, 22 kDa Protéines de liaison	GH recombinante 22 kDa 22° SI 98/574 Dilution des étalons dans du sérum humain
TSH	Modification de glycosylation	Productions de nouveaux calibrateurs structurellement et immunologiquement proches de la TSH circulante (travail en cours)
Insuline	Effet matrice	Production d'un nouveau matériel de référence et mise au point d'une méthode de référence (travail programmé par l'American Diabetes Association)

"[Immunoanalysis, from theory to selection criteria in clinical biology]" Chap. 3 Problems and pitfalls in immunoanalysis. Valat C, Sapin R. EDP Sciences 2009.

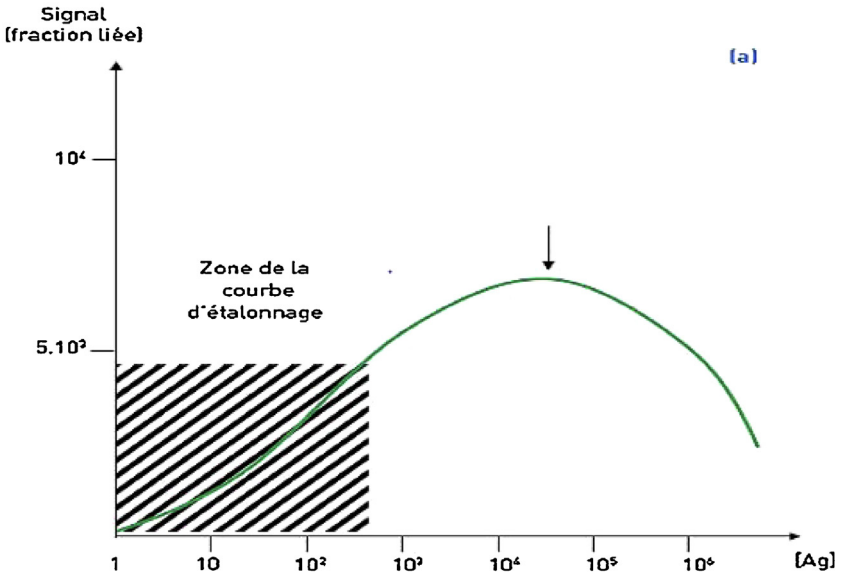

Fig. 2. Hook effect.
Effet croche : représentation de l'effet croche. Quand la concentration en antigène est supérieure à celle indiquée par la flèche, la concentration en anticorps marqué n'est pas en excès par rapport à l'antigène et la quantité de marqueur lié à la phase solide décroît.
"[Immunoanalysis, from theory to selection criteria in clinical biology]" Chap. 3 Problems and pitfalls in immunoanalysis. Valat C, Sapin R. EDP Sciences 2009.

3.3. AB specificity

Although the Ags to be assayed are usually macromolecules expressing many epitopes, Ab specificity cannot be ignored, since the prerequisite for immunoassay is to have an Ab specific for the Ag under assa. The presence within the biological sample of molecules that are structurally related to the Ag to be assayed (drugs, metabolites) may lead to cross-reactions. The main problems with Ag specificity in current immunoassays are listed in Table 2.

3.4. Analytical interference

The sample may be contaminated by the analyte or by a substance cross-reacting with the analyte. The first possibility is inter-sample contamination, found in automated systems for which there is a problem of rinsing the sampling needle and where one sample containing very large amounts of Ag (tumour markers, hCG) may contaminate the next sample. Similarly, if the next sample contains no measurable amount remaining traces can be Ag carried over from the previous sample (hormone replacement therapy or undetectable prostate specific antigen [PSA]). The second pitfall is experimenter contamination, e.g. oestradiol (nasal or transdermal hormone replacement therapy), testosterone (gel treatment), or salivary cortisol (hydrocortisone cream). Thirdly, contamination can result from the presence of signal-modifying substances such as enzyme inhibitors, fluorophores, or in certain cases, the gel used to separate collection tubes. Freedom from interference must also be ensured for radioimmunoassays of samples from patients treated with radioactive iodine (^{123}I or ^{131}I).

Interference from binding proteins can be found in assays of thyroid hormones, steroids (cortisol, oestradiol, progesterone, testosterone, etc.), GH, IGF-1, and for vitamin D in both circulating unbound and transport protein bound forms. For the determination of total forms, it is necessary to ensure complete dissociation of the bound fraction. For the determination of free forms, care should be taken to avoid upsetting the bound/unbound balance.

Table 2
Problems of specificity in current immunoassays.
Principaux problèmes de spécificité dans les immunodosages actuels.

Analyte	Molecules able to cross react with the analyte
Competitive assay	
Cortisol (plasma)	11 deoxycortisol, 21-desoxycortisol, prednisolone, methylprednisolone, prednisone metabolised to prednisolone
Cortisol (urine)	Corticoidsteroids, metabolites of conjugated and unconjugated cortisol
T3	Triiodothyroacetic acid, diclofenac
Testosterone	Dihydrotestosterone, DHEA sulphate
17α-OH progesterone	17 OH pregnenolone sulphate (neonatal period)
Aldosterone	Tetrahydroaldosterone gluconide (renal failure)
Oestradiol	Oestrone (hormome replacement therapy)
Progesterone	Dihydroprogesterone (oral treatment by micronised progesterone)
Digoxin	Spironolactone
Medications	Drugs
Drugs	Medications
Immunometric assay	
Parathormone	Fragments, PTH 7-84
Insulin	Proinsulins, pharmacological analogues
ACTH	Cleaved forms
Calcitonin	Procalcitonin

Widespread use of these techniques should not obscure the fact that immunoassays can be flawed in particular circumstances. In such cases, the error generally affects only one given patient and can be attributed to analytical interference. These are probably the most difficult problems to identify [6].

Immunoassays allow highly sensitive assays, but are inherently prone to interference between Abs introduced by the sera of patients and the Ab reagents used in the assay system. Many studies have been conducted to understand and correct for these interferences. Moreover, some of these sources of interference persist in a random manner, are patient-dependent, and occur at levels that may distort the results dramatically. Thus, if an immunoassay result is discordant with clinical observations, or variable between two different assay techniques, the culprit may well be heterophile antibody (HA) interference. Many publications describe these different assay problems. Prescribers and biologists should be as aware of laboratory interference as they are of drug-drug interference.

Most cases of interference to the immune response upon which the immunoassays are based involve HAs. HAs denote human Abs present in the blood and directed against multiple Ags. HAs are poorly specific, recognising Abs from different species (here considered as Ags). Thus, they can interact with the animal Abs used in the assays. HAs may target the Ag-binding site of the Ab (idiotype), e.g. the constant domain of immunoglobulins (Fab'2 fragments, Fc). By binding to Abs in the assay system, they mimic the analyte to be measured causing interference that falsely augments the result in systems such as the sandwich assay (most frequently encountered today), or inversely, diminish the signal, leading to underdosing in other systems such as immunometric tests (Fig. 3).

Usually the reaction proceeds according to situation [A]: the Ag to be assayed is sandwiched between the capture Ab and the revelator Ab. The signal is proportional to the binding of the revelator Ab on the complex, i.e., the amount of Ag present in the sample. If an interfering Ab is present: two scenarios are possible. As illustrated in situation [B], a heterophile Ab will bridge the capture Ab and the revelator Ab regardless of whether the Ag to be assayed is present or not, and thus give a falsely high result. In situation [C], a heterophile Ab recognises, even only partially, the capture Ab, but has no site for the attachment of the revelator Ab and thus gives a false-negative result.

The presence of HAs in serum can come from interspecies immunisation (so-called allotypic immunisation) that occurs among people who are routinely in contact with animals (typically animal house technicians), or patients exposed to non- or partially-humanised Abs (immunotherapy, targeted Ab therapy, molecular imaging using Abs, etc). This group includes all human anti-animal Abs, the most common being anti-mouse Abs called human anti-mouse antibodies (HAMA). These Abs generally exhibit cross-reactivity between species and are capable of binding variably to Abs of equine, bovine, ovine, and other origins. They can interfere in several assay systems that use Ab reagents from different species. HAs are also observed in immune disorders such as autoimmune diseases. For example, the rheumatoid factor observed in patients with rheumatoid arthritis consists of a self-Ab of isotype M, G or A specific to the Fc fragment of human IgG isotype [7]. HAs are found in the course of some infections, and their presence may even be a useful biomarker for diagnosis, as is the case in infectious mononucleosis. Conventionally, Abs directed against the analyte (thyroglobulin, prolactin, insulin, troponin, T3, T4, GH, PTH, TSH, LH, FSH, etc.) are distinguished from Abs directed against a dosing reagent (HAMA, rabbit, avian or streptavidin, fluorescein, ruthenium, gelatin, etc.).

Studies have shown that HAs can be present in 40% of patient sera [8]. Due to their low affinity and the addition of blocking agents to the reagents, interference generated by these heterophile Abs usually remains marginal in frequency and in intensity. Nevertheless, they are not less significant and difficult to detect.

Interference may have serious consequences for assays where test sensitivity is crucial, e.g. monitoring protocols (PSA for prostate cancer, Tg for differentiated thyroid cancer, AFP and β-HCG for testicular cancer). A usually minimal level of interference may make a negative result positive or negate a positive result.

HA interference must be considered whenever an immunoassay result is discordant with clinical observations or between two assay methods. HA interference can be removed

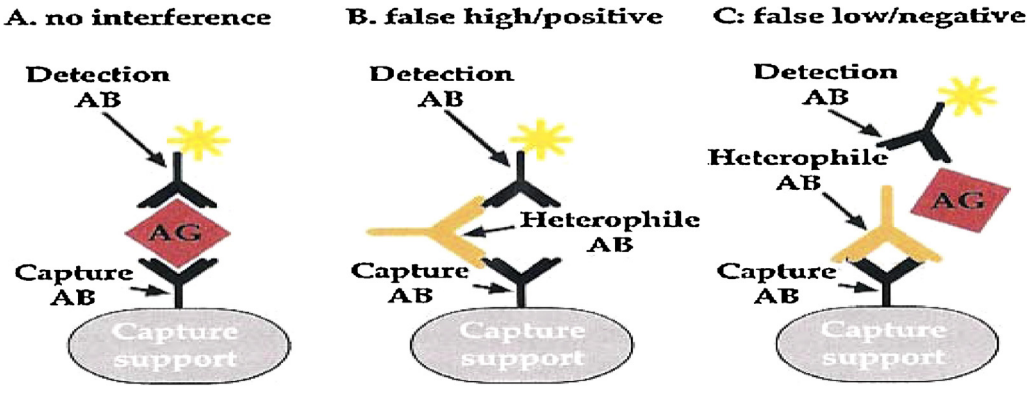

Fig. 3. Diagram of the various interference processes.
Schéma des différents processus d'interférence.

or limited by diluting the test sample (no linear relationship between dilution and assay result). Methods of HA extraction or capture (Heterophilic Blocking Tube [HBT], Scantibodies Laboratory Inc., Santee, California USA) can also be used. Assay results obtained before and after this action will be different. Finally, it should be noted that HAMA assay kits are available. The special case of Tg is worth mentioning. Tg results have to be validated with an immunometric method to make sure they have not been lowered by self-Ab anti-Tg interference. Therefore, when monitoring differentiated thyroid cancer, Tg assay must be interpreted in combination with a search for anti-Tg Abs, recognising the still imperfect concordance of results produced by different anti-Tg Ab kits [9].

4. Consequences for patient management

The result of an incorrectly abnormal immunoassay, whether lowered or raised as is usually the case, suggests a medical condition and can have unfortunate consequences. Similarly a false-negative can be detrimental to proper disease management. A false-negative hCG result for a patient with choriocarcinoma and a false-negative Tg result for a patient with recurrent thyroid cancer are two examples.

In many cases, the false alarm generates a requirement for other assays or further investigations to revisit the diagnosis. However, the stress of an erroneous result is not negligible and additional investigations have a cost (MRI following a false diagnosis of hyperprolactinaemia due to macroprolactin) and are sometimes invasive, such as fine-needle aspiration for a false-positive calcitonin result, coronary angiography for a false-positive troponin assay, treatment with ^{131}I due to a false-positive Tg test [10,11].

In other cases, the result can lead to the initiation of drug treatments that later prove unwarranted, e.g. anti-prolactin or anti-thyroid treatment for false increase in prolactin or thyroid hormone, or thyroid hormone treatment of a pregnant woman in the 2nd or 3rd trimester because results were not interpreted with adequate standards for free T4. An interference-related false-positive oestradiol result can lead to unwarranted discontinuation of an ovarian stimulation cycle. False-positive hCG results incorrectly indicating a tumour or pregnancy can have unfortunate consequences: initiation of chemotherapy, exclusion from a drug protocol or a kidney transplant.

Finally, a false result can be the source of unwarranted surgery. The most frequent examples involve false-positive hCG results suggesting choriocarcinoma or trophoblastic disease and false-positive testosterone results suggesting a tumour.

Coordination between biologists and clinicians is necessary to limit the adverse consequences of a false laboratory result. Cooperation with industrial research laboratories is increasingly necessary due to the widespread use of closed automatic systems.

A literature search conducted in 2012 focused on interference in PSA assay. Over 3.5 million PSA assays were billed to the French health insurance scheme in 2010 (source www.ameli.fr) that showed a prevalence of 0.3% interference due to HAs in PSA assays [12]. Most of the situations described above are clinically documented. In one report, falsely elevated PSA used for early diagnosis prompted a change in the therapeutic approach [13]. In others examining post-therapeutic situations (after prostatectomy or after radiotherapy), falsely elevated levels led to a suspicion of recurrence [14–16]. A mismatch between an immeasurable PSA level in early diagnosis with measurable LiPSA alerted concern about the relevance of the PSA results [17,18].

5. Conclusion

Analytical biologists must know the limitations of their assay methods and must have access to various measurement techniques for their parameters, if necessary through collaboration with colleagues. Dilutions should be made for all doubtful cases since interference is rarely linear; moreover, dilutions eliminate the Hoock effect. In extreme cases, referral to a specialised unit is preferable.

Clinicians should also take care to inform biologists when a patient's serum is potentially subject to analytical problems, for example the Hoock effect in case of very advanced stage cancer or a very large adenoma, presence of anti-insulin Abs in patients treated with insulin, or in an autoimmune context with a strong suspicion of HAs. Clinicians should alert the laboratory when test results appear incongruous for the clinical context.

It is only through dialogue based on mutual trust between the different partners, analytical biologists, clinicians and industry, that diagnostic errors and their consequences can be avoided.

Disclosure of interest

The author declares that she has no conflicts of interest concerning this article.

Acknowledgements

This article is the result of a collaboration between members of the Société Française de Médecine Nucléaire (SFMN) specialised biology group who all participated actively in updating the only book available in French on immunoanalysis published in 2009 [Immunoassay: from theory to selection criteria in clinical biology] EDP Sciences 2009 ISBN: 978-2-7598-0432-0 (in French).

We thank Dr Alison Foote (Grenoble Clinical Research Centre) for translating the manuscript.

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