

Consensus of the French Endocrine Society

Fast test: Clinical practice and interpretation^{☆,◇}

Épreuve de jeûne : réalisation pratique et interprétation

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1. Guidelines methodology

A list of questions to be addressed was first drawn up.

Articles for analysis were retrieved using the following keywords or combinations of keywords: hypoglycaemia, insulinoma, fast test, insulin, C-peptide, proinsulin, beta-hydroxybutyrate (bOH), oral glucose tolerance test, mixed meal, and glucagon stimulation test; the search limits were: English, French. We also made use of the various textbooks and guidelines available. This literature review led to the guidelines laid out below. The level of evidence is weak, as the study populations in question (patients and controls) were all small.

2. Rationale and objectives of the fast test

2.1. Guideline

The fast test is the reference exploration for hypoglycaemia in non-diabetic subjects, and is to be performed in first intention. There is no present evidence in the literature for using alternative

examinations, such as oral glucose tolerance test (OGTT), the mixed meal or glucagon stimulation test, as first-line tests; they are rather to be used in case of doubtful diagnosis following a well-conducted fast test.

2.2. Present state of knowledge

The fast test is the reference exploration for hypoglycaemia in non-diabetic subjects. It is a physiological test providing a general exploration of the integrity of all the various biological systems involved in adaptation to intense and prolonged restriction of calorie input. It has a two-fold objective.

Firstly, it serves to demonstrate objectively the existence of organic hypoglycaemia in patients with suggestive symptomatology but in whom, for practical reasons, no critical venous hypoglycaemia can be shown. An essential point is that a normal fasting-test result does not rule out functional or post-stimulation hypoglycaemia.

Secondly a hypoglycaemia-positive fast test enables the underlying aetiological mechanisms to be determined [1–6].

Several alternative tests have been proposed, to allow patients to avoid the hardship of the fast test. Cohn's diet, the tolbutamide test, the leucin test and the C-peptide suppression test by insulin have been used in suspicion of hypoglycaemia [7,8], but have not been re-assessed recently: there are no recent studies to validate these tests or determine their exact sensitivity and specificity.

The C-peptide suppression test alone has been re-assessed more recently [7]. It is hard to interpret, in as much as age, sex and body mass index have to be taken account [7]. Its diagnostic value is hard to judge, since only eight cases of insulinoma were included in the study by Service et al.

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[☆] Hypoglycaemia in non-diabetic patient.

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These tests were not adopted in the 2009 Endocrine Society Clinical Practice Guideline [2]. There are presently no grounds for recommending them.

A glucagon stimulation test, independent of fast test, may on the other hand be of interest (Appendix 1). Glucagon is a beta-cell secretagogue, and it has long been known, and recently confirmed, that the glucagon stimulation test can, independently of the fast test, reveal hyperinsulinemic hypoglycaemia and diagnose insulinoma where fasting-test results appeared negative [9,10]. There have, however, not been enough studies for the glucagon stimulation test to be justified in first intention before the fast test.

3. Practical realization of the fast test

3.1. Guidelines

The present guidelines for implementing the fast test are based on the literature discussed below and are summarised in Appendix 2. The fast test consists in a complete fast, although with sufficient non calorie fluids (water, infusion, etc.) [1,2]. Non-essential medication should be interrupted.

Extending the fast for 72 hours maximises sensitivity.

During the fast, the following provisions are recommended:

- capillary glycaemia monitoring every 2 hours;
- blood-sampling with systematic venous glycaemia assay every 6 hours until values fall below 0.60 g/L (3.3 mmol/L), then every 2 hours; also to be performed in case of clinical events;
- insulin and C-peptide assay on all samples with venous glycaemia less than 0.45 g/L (2.5 mmol/L);
- proinsulin and beta-hydroxybutyrate (bOH) assay on all samples with venous glycaemia between 0.45 and 0.60 g/L (2.5–3.3 mmol/L);
- exploration for sulfonylureas and meglitinide when glycaemia falls below 0.60 g/L (3.3 mmol/L);
- exploration for anti-insulin antibodies in suspected dysimmunity, in case of myeloma, in case of significant insulin elevation with C-peptide not corresponding to hypoglycaemia, or when no obvious insulinoma is found on imaging despite a biological aspect of hyperinsulinemic hypoglycaemia without detectable sulfonylureas and meglitinide; the assay may be conducted outside of any hypoglycaemic episode;
- no systematic exploration for anti-insulin-receptor antibodies.

In practice, it is therefore recommended to take a full range of samples each time and to ask the laboratory to assay insulin, C-peptide, proinsulin, bOH, sulfonylureas and meglitinide only in the light of the venous glycaemia results (Table 1).

The fast test should be discontinued in case of:

- severe clinical signs: loss of consciousness, convulsions, onset of psychiatric signs. The fast may be stopped without waiting for the venous glycaemia results. However, a venous sample should be taken to assay venous glycaemia, insulin, C-peptide and, if possible, proinsulin and plasma bOH before administering any oral or intravenous glucose;
- suggestion of hypoglycaemia or the presenting symptomatology associated with venous glycaemia less than 0.45 g/L (2.5 mmol/L). Unlike American expert opinion, we advise waiting until venous glycaemia reaches 0.45 g/L (rather than 0.55 g/L) if clinical signs are minor, in order to enhance test specificity;
- venous hypoglycaemia less than 0.40 g/L even if asymptomatic, as, if the glycaemia technique is reliable, subjects never reach this level during a fast test [1,11]. In case of chronic spontaneous hypoglycaemia, clinical sensitivity to hypoglycaemia has been reported to be reduced, as in diabetic patients, and low glycaemia may then be asymptomatic.

In contrast to the American consensus, aborting the fast in the light of plasma bOH findings is not recommended, as some insulinoma patients have been reported to show fasting plasma bOH greater than 2.7 mmol/L [10,12]. The fast may, however, be very poorly tolerated by certain healthy subjects due to severe ketosis with clinical signs of nausea and vomiting; in such highly symptomatic patients, it is reasonable to stop when plasma bOH rises above 2.7 mmol/L [5].

3.2. Present state of knowledge

3.2.1. Tests to perform ahead of the fast test

In suspected adrenal insufficiency, a preliminary adrenocortical function test, such as the Synacthen test, should be performed [2].

3.2.2. Practical conditions for patient

The fast test consists in total fasting, but with sufficient non calorie fluids (water, infusion, etc.) [1,2]. Non-essential medication should be interrupted.

Table 1

Summary in sampling condition. Sampling conditions are summarised in the following Table and detailed in Appendix 3.

Analysis	Tube	Special precautions
Glycaemia	Sodium fluoride	Plasma sampling, no capillary or serum samples, quick transfer to lab
Insulinemia	Dry tube or on anticoagulant according to technique	No hemolysis
Serum or plasma C-peptide	Dry tube or on anticoagulant according to technique	In case of doubt, check renal function (creatinine), quick transfer to lab
Proinsulinemia	Dry tube or on anticoagulant according to technique	
Beta-hydroxybutyrate (bOH)	To be decided with the biologist	

Given the risk of severe hypoglycaemia during the test, it should be performed under hospital admission, with medical and paramedical supervision [1,4,5].

The patient should stay active throughout, but within reach of the paramedical and medical staff, to allow testing in case of onset of hypoglycaemia symptoms [2,5].

3.2.3. Sampling

Samples should be taken during the test to assay venous glycaemia, insulinemia and C-peptide, and also proinsulin and plasma bOH, which should be assayed systematically every 6 hours so long as venous glycaemia exceeds a threshold of 0.60 g/L and every 1 or 2 hours once it falls below [1,2,13]. The same sampling procedure should be applied in case of clinical signs suggestive of hypoglycaemia.

At the end of the fast test, if venous glycaemia has fallen below 0.60 g/L, plasma hypoglycaemic sulfonylureas and anti-insulin antibodies should also be assayed [2]. Exploration for anti-insulin-receptor antibodies, on the other hand, need not be systematic.

3.2.4. Duration

Classically, fast tests last 72 hours [1,5]. There has been shown to be no diagnostic benefit in continuing to 96 hours' fasting [5]. Certain authors have suggested shorter 48-hour tests [4,14]. A large majority of insulinoma patients show hypoglycaemia within the first hours of fasting [4,11,15]: 65–85% within 24 hours [4,5,16], and 90–94% or even 100% [4] within 48 hours; however, it has also been reported that insulinoma patients showed hypoglycaemia only at day 3 of fasting [17–19].

The criteria for ceasing the fast before 72 hours are clinical and based on venous glycaemia. Several recommendations are to be found in the literature.

It was initially advised to discontinue for glycaemia less than 0.45 g/L (2.5 mmol/L) in clinically symptomatic patients or less than 0.40 g/L (2.2 mmol/L) in asymptomatic patients [1].

More recent recommendations [2] set the following criteria:

- Whipple's triad;
- venous glycaemia less than 0.55 g/L (3.0 mmol/L) with well-documented history of Whipple triad;
- bOH greater than 2.7 mmol/L.

4. Interpretation of fast test

4.1. Guideline

Diagnostic strategy is presented in Appendix 4.

4.1.1. Important preliminaries

Before implementing the thresholds laid out below, it is important to have them validated by one's own medical biology laboratory: they were validated using the following assay methods and kits, in patients free of renal or hepatic insufficiency and of history of partial pancreatectomy:

- immunoradiometric assay (IRMA) or immunochemiluminometric assay (ICMA) for insulin, notably using the Bi-insulin IRMA or ADVIA Centaur kit for insulin [11];
- ICMA for C-peptide, notably using the ADVIA Centaur kit [1,11];
- ICMA or radioimmunity assay for proinsulin, notably using the Proinsulin RIA kit [1,11,20,21];
- automatic kinetic assay for bOH [11,16,22].

Guidelines for the various assay methods are summarised in Appendix 3.

4.1.2. Glycaemia less than 0.45 g/L (2.5 mmol/L)

4.1.2.1. *Concomitant with insulin less than 3 mIU/L and C-peptide less than 0.6 ng/mL.* Insulinemia less than 3 mIU/L associated with serum C-peptide less than 0.6 ng/mL indicates either non-insulin hypoglycaemia or injections of insulin-analogue not recognised on insulinemia assay. Diagnosis of healthy subjects should be made only with great caution in such a case.

4.1.2.2. *Concomitant with insulin greater or equal to 3 mIU/L and C-peptide greater or equal to 0.6 ng/mL.* Insulinemia greater or equal to 3 mIU/L and/or C-peptide greater or equal to 0.6 ng/mL indicate insulinoma or nesidioblastosis. However, insulinemia below the 3 mIU/L threshold on specific insulin assay does not rule out such diagnosis.

The main differential diagnosis concerns occult intake of hypoglycaemic sulfonylureas or meglitinide, revealed by specific research on a sample taken under hypoglycaemia, which is the only biological differentiation from insulinoma or nesidioblastosis.

The second differential diagnosis concerns hypoglycaemia by anti-insulin antibodies, where insulinemia greatly exceeds 3 mIU/L and is associated with C-peptide greater than 0.6 ng/mL; anti-insulin antibody assay is positive.

4.1.2.3. *Concomitant with insulin greater than 3 mIU/L and C-peptide less than 0.6 ng/mL.* Insulinemia greatly exceeding 3 mIU/L associated with C-peptide less than 0.6 ng/mL indicates injection of exogenous insulin.

Certain insulin-analogues (lispro, aspart, glargine, etc.), however, are not recognised on certain insulin assays, and insulinemia may also be low; the presentation thus mimics non-insulin hypoglycaemia.

Exceptionally, the presence of anti-insulin-receptor antibodies [23] or an insulin-receptor mutation [24] may induce a similar presentation.

4.1.3. Glycaemia between 0.45 g/L and 0.60 g/L (2.5–3.3 mmol/L)

At this level of glycaemia, neither insulin nor C-peptide is of sufficient diagnostic value, and bOH and proinsulin results are essential.

4.1.3.1. *Concomitant with proinsulin less than 5 pmol/L and plasma bOH greater than 2.7 mmol/L.* Proinsulinemia less than

5 pmol/L associated with plasma bOH greater than 2.7 mmol/L corresponds to adapted glycaemia results, and is found in healthy subjects.

4.1.3.2. Concomitant with proinsulin greater than 22 pmol/L and plasma bOH less than 2.7 mmol/L. Proinsulinemia greater than 22 pmol/L or plasma bOH less or equal to 2.7 mmol/L indicates insulinoma or nesidioblastosis.

4.1.3.3. Concomitant with proinsulin between 5 and 22 pmol/L. Here, the level of evidence for or against diagnosis of insulinoma or nesidioblastosis is weak, as the literature is lacking in data. Insulinoma or nesidioblastosis can thus not be ruled out, especially if the clinical presentation is highly suggestive of organic hypoglycaemia and/or if bOH is lower than 2.7 mmol/L.

4.1.4. Glycaemia greater than 0.60 g/L (3.3 mmol/L)

Absence of glycaemia less than 0.60 g/L usually rules out hypoglycaemic spells; any actual hypoglycaemic spells thus needs reconsidering, to rule out non-hypoglycaemic aetiologies. In case of strong clinical suspicion of organic hypoglycaemia, however, complementary tests may be performed.

4.2. The present state of knowledge

4.2.1. Important preliminaries

The thresholds given below are to be used with caution: they were validated by particular teams, using particular kits and standards.

It should also be borne in mind that these thresholds were established in patients free of any other pathology liable to impact insulin resistance, insulin secretion or the metabolism of insulin and peptides co-secreted by islet of Langerhans beta-cells, and should therefore be applied with caution in case of known associated pathology. They have, for example, been shown to be non-applicable in case of renal insufficiency [25], where plasma bOH assay and the glucagon stimulation test are recommended for diagnosing hyperinsulinemic hypoglycaemia. The present thresholds have not as yet been validated in hepatic insufficiency or after partial pancreatectomy.

They may also prove erroneous in certain exceptional cases of hyperinsulinemic hypoglycaemia by nesidioblastosis [11,26]. Notably, in certain genetic forms of nesidioblastosis, inappropriate insulin secretion has been reported for glycaemia levels equal to or less than 0.45 g/L but with suppression of insulin, C-peptide and proinsulin at lower glycaemia thresholds [27].

4.2.2. Glycaemia

The American consensus set a glycaemia threshold at 0.55 g/L (3.0 mmol/L) [2], for which insulin, C-peptide and plasma proinsulin were suppressed in a population of 33 healthy volunteers (16 male, 17 female) [28].

This 0.55 g/L threshold is, however, debatable as regards diagnosing either organic hypoglycaemia or hypoglycaemia related to endogenous hyperinsulinism. Few healthy subjects reach a threshold of 0.45 g/L (2.5 mmol/L) during the fast test, while some may reach venous glycaemia levels below 0.55 g/L

[1,11,29]. Furthermore, studies validating insulin, C-peptide and proinsulin thresholds in an insulinoma population applied a glycaemia threshold of 0.45 g/L (2.5 mmol/L) [1,11,21]. A threshold of 0.9 ng/mL rather than 0.6 ng/mL was recommended for C-peptide by Marks et al. for a glycaemia threshold of 0.5 g/L [3]. Finally, the value of insulin, C-peptide and proinsulin thresholds for diagnosing hypoglycaemia by inappropriate insulin secretion has been shown to be better when glycaemia is less than 0.45 g/L, rather than using a threshold of 0.60 g/L (3.3 mmol/L) or even 0.50 g/L (2.75 mmol/L) [11,21].

4.2.3. Insulin

For immunometric insulin assay, usually using monoclonal antibodies without significant cross-reaction with proinsulin, the American consensus recommended an insulinemia threshold of 3 mIU/L for hypoglycaemia less than 0.55 g/L (3.0 mmol/L) [2].

Other authors recommend a threshold of 3 mIU/L for hypoglycaemia less or equal to 0.45 g/L (2.5 mmol/L) [13]. Insulinemia greater or equal to 3 mIU/L for venous glycaemia greater or equal to 0.45 g/L confirms a diagnosis of organic hypoglycaemia with a specificity of 100% [11]; insulinemia less than 3 mIU/L for the same glycaemia level, on the other hand, does not rule out organic hypoglycaemia, 11% to 35% of insulinoma patients failing to reach this threshold [11].

Marks et al. recommended a threshold of 5 mIU/L for glycaemia less than 0.5 g/L [3], but on the basis of competitive insulin assay, which is no longer widely used.

4.2.4. C-peptide

C-peptide has no biologic activity, yet serum or plasma assay has a better diagnostic value than insulinemia. Results are expressed as pmol/L or ng/mL; pmol/L is converted into ng/mL by multiplying by 0.003.

The American consensus sets the C-peptide threshold at 0.6 ng/mL for hypoglycaemia less than 0.55 g/L [2]. Other authors recommend 0.6 ng/mL for hypoglycaemia less or equal to 0.45 g/L (2.5 mmol/L), as this has almost 100% sensitivity and specificity in diagnosing hypoglycaemia by inappropriate insulin secretion [13]: only 1 out of 33 patients with hypoglycaemia by inappropriate insulin secretion failed to reach this threshold [11].

The 0.9 ng/mL threshold set by Marks et al. for glycaemia less than 0.5 g/L seems less effective for diagnosis, notably in terms of sensitivity [3].

4.2.5. Proinsulin

The diagnostic interest of proinsulin assay, compare to insulin and C-peptide assay alone, is well-established for glycaemia in the 0.45 to 0.60 g/L range during fasting, and also for glycaemia greater than 0.60 g/L or less than 0.45 g/L.

Proinsulinemia exceeding 22 pmol/L has been shown to diagnose hyperinsulinemic hypoglycaemia with 100% specificity and 74% sensitivity [11] for glycaemia in the 0.45 to 0.60 g/L range, in which both insulinemia and C-peptide fail to distinguish patients from controls (85% sensitivity for both) [20]. Proinsulinemia less than 5 pmol/L in this glycaemia range is adapted and corresponds to control findings, especially when

plasma bOH exceeds 2.7 mmol/L. Proinsulinemia between 5 to 22 pmol/L for glycaemia of 0.45 to 0.60 g/L is harder to interpret: according to the Endocrine Society consensus [2], proinsulinemia greater or equal to 5 pmol/L for glycaemia less than 0.55 g/L indicates hyperinsulinemic hypoglycaemia with 68% to 78% specificity [21]; complementary results (bOH, glucagon stimulation test) should then be taken into account.

Proinsulin assay may also be useful in glycaemia equal to or less than 0.45 g/L, where its diagnostic reliability may be slightly better than that of C-peptide (100% versus 97% sensitivity, respectively, in diagnosing hypoglycaemia by inappropriate insulin secretion), with a threshold of 5 pmol/L [11]. At this level of glycaemia, the 22 pmol/L threshold previously recommended by Hirshberg et al. [4] shows poor sensitivity (18% of patients failing to reach the 22 pmol/L threshold with hypoglycaemia less or equal to 0.45 g/L) without improved specificity [11].

Finally, proinsulinemia exceeding 22 pmol/L after an overnight fast (not the prolonged 72 hour-fast test) in young non-obese subjects free of pathology liable to affect insulin sensitivity or proinsulin metabolism was shown to be a very strong indicator of insulinoma, even without associated hypoglycaemia [4,11].

4.2.6. *Beta-hydroxybutyrate (bOH)*

Fasting-test plasma bOH assay has two objectives: to check compliance, and to rule out hypoglycaemia by inappropriate insulin secretion without waiting for glycaemia to fall below 0.45 g/L (2.5 mmol/L). Plasma bOH exceeding 2.7 mmol/L during the fast test for glycaemia in the 0.50 to 0.60 g/L (3 to 3.3 mmol/L) range rules out organic hypoglycaemia by inappropriate insulin secretion with sensitivity and specificity approximating 100% [21,30], which neither insulinemia nor plasma C-peptide can do [30]. For glycaemia less than 0.50 g/L, plasma bOH again is of great diagnostic value [21]: 100% sensitivity and specificity, although clinically contradictory cases have been reported [9,10]. Moreover, available data indicate that bOH does not exceed 2.7 mmol/L under fasting in case of Insulin Growth Factor (IGF) secretion [2,13], which extends its diagnostic applications beyond insulinoma, providing a contribution not available with insulin and C-peptide assay alone.

4.2.7. *Miscellaneous ratios*

The insulin/glycaemia ratio, Turner index, proinsulin/glycaemia ratio and proinsulin/insulin ratio do not seem to provide any better diagnostic contribution than the various parameters detailed above [1,11] and were not included in the Endocrine Society consensus [2]; there are presently no grounds for recommending them.

4.2.8. *Insulin secretagogue assays*

Hypoglycaemic sulfonylureas and meglitinide assay is the only biological means of detecting their occult intake during fasting: other findings fail to differentiate from insulinoma or nesidioblastosis.

The assay should detect as wide as possible a range of insulin secretagogues, to avoid false negatives. Exploration for hypoglycaemic sulfonylureas intake should ideally be performed on a

blood sample taken under or as quickly as possible after hypoglycaemia; otherwise, factitious hypoglycaemia cannot be ruled out with certainty: sulfonylureas are usually rapidly eliminated from the blood after a single intake, with elimination time ranging from 12 to 24 hours depending on the molecule, so that high-sensitivity techniques are required; elevated specificity is, obviously, also important.

4.2.9. *Anti-insulin antibodies*

Expected reference values for free and total anti-insulin antibodies are generally less than 6–7%.

Presence of anti-insulin antibodies is not easy to interpret: when animal insulin was on the market, it often pointed to administration of exogenous insulin; nowadays, however, most patients taking exogenous insulin no longer show anti-insulin antibodies, probably because modern insulin is of low antigenicity. Presence of anti-insulin antibodies may thus indicate auto-immune hypoglycaemia [31]; but this is a rare entity, with less than 200 cases reported in the literature, and those mainly being of Japanese origin [32] or multiple myeloma patients [33,34]. Anti-insulin antibodies may also be found in healthy subjects or even in insulinoma patients [35]. Auto-immune hypoglycaemia should therefore not be diagnosed too hastily.

4.2.10. *Anti-insulin-receptor antibodies*

To date, anti-insulin-receptor antibodies have been implicated in less than 200 hyperinsulinemic hypoglycaemia patients in the literature worldwide; the context, moreover, was often particular, involving disseminated erythematous lupus, scleroderma, primary biliary cirrhosis, celiac disease, severe insulin resistance syndrome, etc. [23].

5. Complementary tests in case of diagnostic doubt in the fast test

5.1. *Guideline*

In patients with apparently normal 72-hour fast test but presenting mainly postprandial hypoglycaemic spells that is clinically highly suggestive of organic hypoglycaemia, complementary testing is possible. These tests are thus of marginal application, as such conditions are met by only a minority of patients.

These tests comprise a glucagon stimulation test at the end of fast (Appendix 1), mixed meal, or oral glucose tolerance test using 75 g glucose (tested hourly for 5 hours).

5.2. *Present state of knowledge*

In patients with apparently normal fast test but presenting malaise that is clinically highly suggestive of organic hypoglycaemia, complementary testing is possible. Certain insulinoma or nesidioblastosis patients may exceptionally have normal fasting-test results (less than 1% of insulinomas) [5].

5.2.1. *End-of-fast glucagon stimulation test*

When end-of-fast glycaemia remains between 0.45 and 0.60 g/L, an end-of-fast glucagon stimulation test may be

recommended (Appendix 1) [1,2,5,13]. An increase of glycaemia below 0.25 g/l after glucagon injection will suggest hypoglycaemia related to endogenous hyperinsulinism or IGF, with 91% sensitivity and specificity of 95–100% [1,21,30].

5.2.2. Oral glucose tolerance test (OGTT) and mixed meal

In case of basically postprandial malaise clinically suggestive of organic hypoglycaemia with normal fast test, two investigations may be made [9,27,36–39]: OGTT using 75 g glucose, and mixed meal; these explorations are not intended to replace the fast test. Exclusively postprandial hypoglycaemia has been reported in association with normal OGTT results and pathological fast test results [40]. These two tests may therefore be reserved for patients showing postprandial hypoglycaemia but without hypoglycaemia at the end of 72-hours' fast test.

In these selected patients, is OGTT or mixed meal preferable? The sensitivity of the former in diagnosing insulinoma is poorly known, but its specificity is probably very low: 10–46% of subjects showed less than 0.50 g/L or even less than 0.40 g/L venous glycaemia on OGTT [41–45]. One reason may be the liquid nature of the glucose load, absorbed much more quickly and less physiologically than in solid form [46].

The mixed meal is considered preferable by certain authors, as it is supposed to correspond to an everyday-life situation [1,2,47]. Execution, however, has yet to be well-codified. On the recommended design [2], venous glycaemia, insulin, C-peptide and plasma proinsulin are to be assayed every 30 minutes during the 5 hours following the meal. The exact composition of the test meal has never been clearly described, and interpretation is vitiated by the lack of validated threshold values for the assays.

Appendix 1. Glucagon stimulation test procedure

1. Glucagon stimulation test after 72-hour fast: intravenous injection of 1 mg glucagon after 72-hours' fasting. measurement of venous glycaemia 10, 15 and 30 minutes post-injection.
2. Glucagon stimulation test remote from fast test: intravenous injection of 1 mg glucagon remote from fast test. measurement of venous glycaemia, insulinemia, C-peptide and proinsulinemia 10, 15 and 30 minutes post-injection.

Appendix 2. Fasting-test procedure

Appendix 3. Sampling and assays

3.1. Glucose

3.1.1. Pre-analytic conditions

Glycaemia should be measured on a venous plasma sample rather than a capillary or serum sample, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) guidelines. Neither capillary glycaemia, measured in total blood, nor serum glycaemia is a reliable indicator of venous plasma glycaemia, and both are less precise [48,49].

Fasting test

Conditions

Patient admitted under medical and paramedical surveillance

Total fast

- Sufficient non-calorific fluid intake
- Interruption of non-essential medication

Duration

72 hours in absence of hypoglycemia

Examinations

Capillary glycemia every 2 hours

Venous glycemia every 6 hours until glycemia less than 0.60 g/L then every 2 hours and in case of clinical symptoms

If venous glycemia less than 0.60 g/L, assay insulinemia, C-peptide, proinsulin, β -hydroxybutyrate + screen for sulfonamides and meglitinides

According to context, anti-insulin antibodies and anti-insulin-receptor antibodies

Glucose levels are 11% higher in plasma than in total blood, entailing a risk of underestimation in serum or capillary samples [50]. The IFCC does provide a conversion coefficient for non-plasma samples (1.11 to convert total blood concentration into plasma concentration) [50]; but this coefficient is dependent on hematocrit [51,52], and it is advisable to measure glycaemia directly from plasma.

The main problem in glycaemia assay is glycolysis induced by cells within the sample. The classic antiglycolytics, sodium fluoride and lithium monoiodoacetate, enhance the long-term stability of glucose but do not prevent glycolysis during the first 4 hours after sampling [53,54]. Glucose stability tests in blood sampled in tubes containing either heparin alone or heparin plus monoiodoacetate found a 9% fall in glucose concentrations during the first 2 hours in both cases [55].

A high concentration of leukocytes in the sample may amplify the effect of glycolysis, inducing an even faster fall in measured glucose levels [53].

There are two means of getting round the problem of glycolysis:

- separate serum from plasma straight after sampling: glucose concentrations remain stable for 8 hours at 25 °C and 72 hours at 4 °C in non-hemolysed serum, separated from cells and sampled under sterile conditions [56];
- or place the tube in ice and separate plasma from cells within 30 minutes [57,58].

These techniques, however, are difficult to implement in ordinary clinical practice in health-care departments, requiring a centrifuge and ice. A recent study showed the interest of using

tubes containing a citrate buffer, sodium fluoride and ethylenediaminetetraacetic acid (EDTA): plasma glucose levels fell by only 0.3% at 2 hours and 1.2% at 24 hours in such tubes conserved at 37 °C [58].

In every case, rapid transfer to the laboratory is advised by the World Health Organisation (Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia). If transfer has to be delayed, conservation at +4 °C minimises glycolysis. Noting the exact sampling time on the accompanying form allows the biologist to formulate any reservations as to the results.

Finally, as is the case with any tube containing a certain quantity of anticoagulant, the tube must be filled sufficiently to prevent the glucose being diluted by the anticoagulant.

3.1.2. Analysis techniques

Venous glycaemia should be measured using a reliable enzymatic technique with glucose oxidase or hexokinase (French national quality control 09BIO1 and 09BIO2 surveys, November 2010). The reference technique is that of hexokinase [59], which, however, is manual and non-automated, so that glucose oxidase is often used instead in clinical routine [60]. Both provide good quality results (coefficient of variation (CV) between 2.7% and 2.8%), although precision varies according to technique, being lowest for glucose oxidase colorimetry (UV end-point method); techniques using hexokinase or glucose oxidase with oxygen consumption or reflectometry all show minimal scatter without bias [61]. There is no consensus as to objectives in terms of the analytic performance of plasma glucose assessment: coefficient of variation (CV) less than 2.2% has been proposed as an intra-lab reproducibility criterion, although this threshold cannot be applied for inter-lab reproducibility [62].

3.2. Insulin

3.2.1. Pre-analytic conditions

For insulin assay, hemolysis must be strictly avoided for results to be valid: erythrocytes contain an enzyme that degrades insulin, causing underestimation of insulinemia in immunometric assay or overestimation in competitive assay [63]. Heparinised plasma sampling is not recommended, giving lower insulin levels than those obtained from serum [64].

3.2.2. Analysis techniques

Appendix 5 presents the various insulin assay kits available in France.

There are highly significant differences between techniques, even though most are calibrated according to the IRP 66/304 standard. There is at present no one reference technique [65,66]. The new 83/500 standard is not yet applied in all insulin assay techniques. The coefficient for converting mIU/L into pmol/L should therefore be used with caution, as it may vary from 6.0 to 7.5. It is thus essential to specify which standard is being applied. Using recent calibrations, 3 mIU/L is equivalent to 18 pmol/L. The latest guidelines from the American Association of Clinical Chemistry (AACC) and American Diabetes Association (ADA) call for real harmonisation of insulin assays (GPP: Good Practice Point) [62]. Insulin assay was for many years performed with

techniques that were not particularly specific to insulin, showing cross-reaction with proinsulin; current assays, however, are highly specific. As certain insulinomas secrete proinsulin rather than insulin, low insulinemia in insulinoma is possible [67].

Anti-insulin antibodies interfere with assay antibodies, artefacting the results, with over- or underestimation depending on the technique; free insulin should then be assayed, with polyethylene glycol precipitation of insulin bound to the patient's anti-insulin antibodies.

3.3. C-peptide

3.3.1. Pre-analytic conditions

C-peptide may be assayed on serum or plasma (EDTA or heparin). Unlike in insulin assay, hemolysis does not affect the results. Creatinemia, on the other hand, may need to be assayed to eliminate renal insufficiency, which would make interpretation difficult (renal elimination of C-peptide). Transfer to the laboratory should be fast; the C-peptide molecule is unstable in serum and plasma, and aliquots should be frozen if assay is not performed within 24 hours.

3.3.2. Analysis techniques

The various C-peptide assay kits available in France are presented in Appendix 6.

C-peptide serum assay is calibrated according to the WHO's IRP 84/510 standard. There are several kits, both competitive and immunometric. Plasma assay shows variable cross-reaction with proinsulin, although less critically than for insulin assay; neither anti-insulin antibodies nor exogenous insulin affect results. Like with insulin, there are important issues of standardisation [68].

3.4. Proinsulin

Intact proinsulin is cleaved into 32–33 split and 65–66 split proinsulin then des-31,32 and des-64,65 proinsulin and finally into insulin and C-peptide [69]. The most abundant forms in serum are intact proinsulin and des-31,32 proinsulin. Two types of proinsulinemia assay exist: intact proinsulin assay, and total proinsulin assay measuring not only intact proinsulin but also its cleavage products; most published thresholds fail to specify the assay type. The commercial kit used in one cited study (Linco Research) reporting a threshold was a “total proinsulin” assay measuring intact proinsulin and the most abundant of the cleavage molecules (des-31,32 proinsulin), with negligible cross-reaction (less than 0.1%) for des-64,65 proinsulin, insulin and C-peptide [11].

Reference values are thus method-dependent.

Finally, some manufacturers allow plasma instead of serum assay, but it should be borne in mind that the resultant values differ greatly (twice that for serum).

3.5. Beta-hydroxybutyrate

3.5.1. Pre-analytic conditions

Beta-hydroxybutyrate (bOH) should be assayed on a peripheral venous sample, and not by semi-quantitative detection in

blood or urine using reactive bands, which detect ketone bodies, a general category including not only bOH but also acetoacetate and acetone: detection is based on a colour reaction between sodium nitroprusside and, mainly, acetoacetate, without detecting bOH.

There is no consensus as to the type of assay tube to be used. Custer et al. reported that serum or plasma obtained by sampling on sodium fluoride or heparin are the media of choice [70]. Uno et al., on the other hand, reported no interference with oxalate, EDTA or citrate [71]. Recent guidelines state that bOH may be assayed in tubes containing heparin, EDTA, sodium fluoride, citrate or oxalate [62]. It is therefore recommended to have sampling conditions specified by the biologist in charge of the assay.

Rapid transfer to the laboratory is essential: delayed separation of plasma from cells significantly reduces bOH levels, whatever the storage temperature, and a maximum interval of 2 hours should be respected [72]. After separation, however, bOH is relatively stable, unlike acetoacetate: a reduction of only 5% was reported after conserving plasma for three days at 4 °C [72]. Finally, ascorbic acid interferes in certain assay techniques, and it should be checked that none was being taken during the fast [62].

3.5.2. Analysis techniques

bOH assay is based on a specific enzymatic method. Most current methods are based on bOH oxidation in acetoacetate by beta-hydroxybutyrate dehydrogenase with NAD⁺ co-substrate and alkaline pH, followed by end-point or kinetics spectrophotometric method. The method initially described involved plasma deproteinisation before the enzymatic reaction, and required 2 hours' manipulation [73]. The small market for this assay means that ready-to-use kits have not been greatly developed by manufacturers. Some teams have adapted clinical biology machines to bOH assay to save time, reduce the required plasma and reagent volumes and improve precision; some report that this allows the deproteinisation step to be skipped [71,72,74]. Even so, "home-made" reagents have to be produced. According to the literature, detection limits and intra-series CVs vary according to the technique, although the former are consistently less than 100 µM and the latter less

than 5% for concentrations approximating 1 mM [72,75]. The European Research Network Inherited Disorders of Metabolism (ERNDIM) external quality control program has an EQC program for bOH: in 2010, the mean bOH value from 101 laboratories was 2.5 mmol/L with an inter-lab CV of 11.5%.

3.6. Anti-insulin antibodies

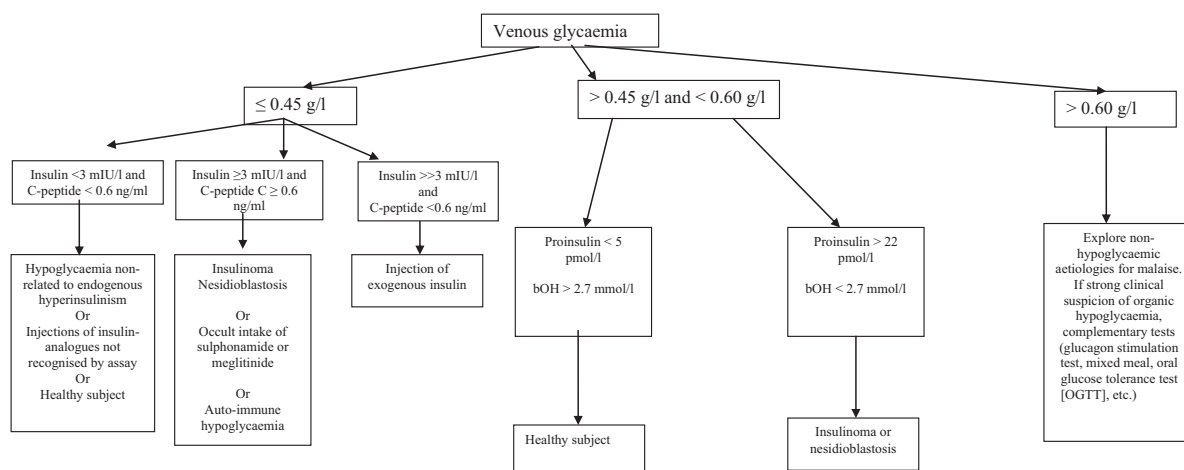
Anti-insulin antibodies are screened for by radioimmunoassay, which correlates with clinical findings more closely than does immunoenzymatic assay. Detection is based on high-lighting specific binding with iodine-125-labeled insulin. IBA's Anti-Insulin RIA kit (Cis BioInternational, Gif-sur-Yvette, France) can assay not only free anti-insulin antibodies (non-complexed with circulating insulin) but also total anti-insulin antibodies (free plus complexed with serum insulin). Free anti-insulin antibody assay is based on radioimmunoprecipitation, whereas total antibody assay requires preliminary immune-complex dissociation in acid medium, insulin adsorption by active carbon and neutralisation.

Samples with hemolysis, hyperlipidemia or fibrin give inexact results.

3.7. Screening for hypoglycaemic sulfonylureas and meglitinide

Screening for hypoglycaemic sulphonamide and meglitinide uses liquid chromatography in certain specialised pharmacology laboratories. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is optimally sensitive and specific [76]. A technique able to identify and quantify 11 oral antidiabetic agents was reported in 2011 [77]; the antidiabetic agents included seven hypoglycaemic sulfonylureas (glimepiride, glibenclamide, gliquidone, glibornuride, glisoxepide, glipizide and gliclazide) and two meglitinides (nateglinide and repaglinide); the detection limit in all cases was 1 ng/mL, enabling detection and quantification at concentrations ranging from sub-therapeutic to overdose.

Appendix 4. Fast test interpretation decision tree



Appendix 5. Insulin assay kits

Assay	Manufacturer	Method
<i>Competitive assay</i>		
INSIK-5	DiaSorin	RIA
Insulin-CT	Cis Bio International-IBA	RIA
Human Insulin-Specific RIA kit	Linco Research-Labodia	RIA
<i>Immunometric assay</i>		
Ins-Irma BioSource	Brahms	IRMA
Insulin IRMA KIT	Beckman Coulter	IRMA
Bi-Insulin IRMA	Cis Bio International-IBA	IRMA
INSI-CTK IRMA	DiaSorin	IRMA
Insulin IRMA	DiaSource	IRMA
Liaison Insulin	DiaSorin	ILMA
Insulin Architect	ABBOTT	ICMA
Access	Beckman Coulter	IEMA
Immulite	Siemens	ICMA
ADVIA Centaur	Siemens	ICMA
Elecsys/Modular	Roche	ICMA/ECLIA
Insulin Elisa Kit	Dako	Elisa
Insulin Auto DELFIA kit	Perkin Elmer	IFMA
AIA Pack	Tosoh Eurogenetics	IEMA

Appendix 6. C-peptide assay kits

Assay	Manufacturer	Method
<i>Competitive assay</i>		
C-peptide	DiaSorin	RIA
C-peptide RIA CT	Diasource	RIA
C peptide	Linco	RIA
<i>Immunometric assay</i>		
C-peptide IRMA kit	Beckman Coulter	IRMA
C-peptide IRMA	Cis Bio International-IBA	IRMA
C-peptide	Diasorin	IRMA
C-peptide ARCHITECT	ABBOTT	ICMA
Access	Beckman Coulter	IEMA
Liaison C-peptide	DiaSorin	ILMA
C-peptide Immulite	Siemens	ICMA
ADVIA Centaur	Siemens	ICMA
Elecsys/Modular	Roche	ICMA/ECLIA
C-Peptide Elisa Kit	Dako	IEMA
C-peptide AutoDELFIA kit	Perkin Elmer	IFMA
AIA Pack	Tosoh Eurogenetics	IEMA

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