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CLINICAL STUDY

Insulin, C-peptide and proinsulin for the biochemical diagnosis of hypoglycaemia related to endogenous hyperinsulinism

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Abstract

Objective: We evaluated the respective value of insulin, C-peptide and proinsulin levels in 33 patients with endogenous hyperinsulinism and in 67 controls to determine the best parameters and thresholds to make or to rule out the diagnosis of endogenous hyperinsulinism.

Results: When blood glucose levels were below 2.5 mmol/l, insulin was <21 pmol/l in 8–35% of the patients and in all controls; C-peptide was >0.2 nmol/l in all insulinomas but not in the nesidioblastosis or in the controls; proinsulin was >5 pmol/l in all patients but not in the controls. When fasting blood glucose levels reached 2.5–3.3 mmol/l, proinsulin was <22 pmol/l in all the controls and >22 pmol/l in 74% of the patients. Proinsulin after an overnight fast was below 22 pmol/l in all non-obese controls and above 22 pmol/l in 73% of non-obese patients.

Conclusion: Proinsulin levels above 5 pmol/l with blood glucose levels below 2.5 mmol/l during a 72 h fast test represent the best criterion for the diagnosis of endogenous hyperinsulinism, reaching 100% diagnostic specificity and sensitivity. Concomitant C-peptide levels above 0.2 nmol/l also make the diagnosis of all our insulinoma patients, not the diagnosis of nesidioblastosis, while insulin levels have much less diagnostic accuracy. Whether proinsulin levels above 22 pmol/l could also make the diagnosis of endogenous hyperinsulinism in part of the patients at the time of fasting blood glucose levels between 2.5 and 3.3 mmol/l or after an overnight fast in non-obese subjects needs further study.

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Introduction

The biological criteria for the diagnosis of fasting hypoglycaemia related to endogenous hyperinsulinism have been challenged within recent years.

They were previously based on the finding of inappropriate serum levels of insulin and C-peptide, and, more recently, of proinsulin, at the time of fasting hypoglycaemia. However, to date, there is no general agreement regarding the diagnostic thresholds to reach in order to conclude that insulin, C-peptide or proinsulin levels are inappropriate. Several years ago, ratios calculated from insulin and blood glucose levels were employed. Then, a 6 mIU/l (43 pmol/l) insulin threshold and a 0.6 ng/ml (0.2 nmol/l) C-peptide threshold concomitant with symptomatic hypoglycaemia below 0.45 g/l (2.5 mmol/l) was established by Service *et al.* (1), while others recommended cut-off values of 5 mIU/l (36 pmol/l) and 0.9 ng/ml (0.3 nmol/l) at the time of hypoglycaemia below 0.5 g/l (2.8 mmol/l; (2)). With the insulin and C-peptide-specific assays devoid of significant cross-reaction with proinsulin which are now in use, a lower insulin threshold of 3 mIU/l (21 pmol/l) was recommended by Service *et al.* (3), but already there are reports of insulinoma patients with insulin levels below 3 mIU/l (21 pmol/l) at the time of symptomatic hypoglycaemia (4–6). Finally, the availability of proinsulin assays led to the use of serum proinsulin thresholds as diagnostic tools; a 5 pmol/l proinsulin cut-off level at the time of hypoglycaemia below 0.45 g/l was recommended by Service *et al.* as a diagnostic criterion (1), but others selected a higher threshold (22 pmol/l (7), 20 pmol/l (8)), for the diagnosis of insulinomas.

In addition, since the proportion of proinsulin secreted by insulinoma cells is generally higher than that secreted by normal β cells, high proinsulin levels were suggested to be a diagnostic tool for insulinomas whatever concomitant blood glucose levels may be. Measurement of proinsulin levels could lead to the diagnosis of insulinoma for blood glucose levels ranging from 2.5 to 3.3 mmol/l during the fast test (9) or when evaluated after an overnight fast, even without concomitant hypoglycaemia (7).

The objective of our study was to assess, in 33 patients with hypoglycaemia related to endogenous hyperinsulinism and in 67 controls, the respective values of serum levels of insulin, C-peptide and proinsulin levels and, among these three parameters now in use and traditional ratios derived from these parameters, to determine the best parameter and

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threshold level to make or to rule out the diagnosis of endogenous hyperinsulinism during the fast test. The second objective of our study was to evaluate whether measurement of proinsulin levels after an overnight fast could allow to make or to rule out diagnosis of endogenous hyperinsulinism in order to avoid the fast test.

Subjects

Patients with endogenous hyperinsulinism

The 33 patients (11 men and 22 women) aged 57±16 years (mean±S.D.; 18–85 years) had a body mass index (BMI) of 25.2±5.1 kg/m^2 (19–42.8 kg/m^2; BMI≥30 kg/m^2 in five patients). In 32 patients, the diagnosis of insulinoma was confirmed by histopathological examination after surgery, and mean insulinoma size was 21±13 mm (8–60 mm). Diagnosis of nesidioblastosis was confirmed in one patient by histopathological examination after a left-sided resection of the pancreas. In this patient, the reported investigations were performed when hypoglycaemic symptoms recurred one year after surgery. Liver and renal functions were normal in all patients.

Controls

The 67 controls (22 men and 45 women) were aged 43±16 years (17–85 years). BMI was 23.3±5.3 kg/m^2 (17.4–44.5 kg/m^2; BMI≥30 kg/m^2 in seven controls). Hypoglycaemia related to endogenous hyperinsulinism was excluded if, during the 72-h fast test, blood glucose did not decrease below 3.3 mmol/l or if serum β-hydroxybutyrate concentrations reached 2700 μmol/l with concomitant blood glucose between 2.5 and 3.3 mmol/l (10, 11). All controls had normal liver and renal functions.

Methods

The 72-h fast test

Patients and controls were fasted for up to 72 h under close medical supervision. Blood glucose, insulin, C-peptide and proinsulin levels were measured after an overnight fast and then every 4 h during the 72-h fast test. Additional samples were taken when blood glucose was <2.5 mmol/l or if clinical symptoms of hypoglycaemia occurred. The fast test was discontinued before 72 h when symptomatic hypoglycaemia with blood glucose below 2.5 mmol/l was presented by the patients (1).

Assays

Blood glucose was measured by standard hexokinase method.

Insulin was measured with an immunoradiometric (IRMA) kit provided by Pasteur Diagnostics, Marnes-la-Coquette (Bi-Insulin Bio-Rad kit) in 17 patients and in 18 controls. There is no significant cross-reaction with proinsulin. The inter- and intra-assay coefficients of variation (CV) are 8.0 and 3.8% respectively, and the smallest detectable level is 1.4 pmol/l (0.2 mIU/l; to convert insulin values from pmol/l to mIU/l, divide by 7.175).

The normal range for fasting serum insulin levels is 14.3–122 pmol/l (2–17 mIU/l).

Insulin was measured with an automated immunochemiluminometric (ICL) assay provided by Bayer Diagnostics (ADVIA Centaur insulin assay) in 28 patients and in 59 controls (12). The normal range for fasting serum levels is 12.2–222.4 pmol/l (1.7–40 mIU/l). The inter- and intra-assay CV are 5.3 and 4.7% respectively. The minimum detectable concentration is 3.6 pmol/l (0.5 mIU/l). There is no significant cross-reaction with proinsulin.

Insulin was evaluated with both IRMA and ICL assays in 12 patients and in 10 controls.

C-peptide levels were measured with the automated immunochemiluminometric method, ADVIA Centaur (Bayer Diagnostics; (12)). The smallest detectable level is 0.02 nmol/l (0.05 ng/ml, to convert C-peptide values from nmol/l to ng/ml, multiply by 3). The inter- and intra-assay CV are 8.3 and 3.7% respectively. The normal range for fasting C-peptide levels is 0.26–0.63 nmol/l (0.78–1.89 ng/ml). There is no significant cross-reaction with proinsulin.

In our study, the thresholds of 21 pmol/l (3 mIU/l; (1)) for insulin-ICL and insulin-IRMA and 0.2 nmol/l (0.6 ng/ml; (1)) for C-peptide were used for comparison.

Proinsulin was measured using the Human Proinsulin RIA kit, provided by Linco Research (St Charles, MO, USA). It yields no significant cross-reactivity with insulin (<0.1%) or C-peptide (<0.1%). The inter- and intra-assay CV are 7.7 and 6.9% respectively. The smallest detectable level is 2 pmol/l. The normal levels for fasting serum proinsulin levels are 7.9±1.5 pmol/l. Two previously recommended proinsulin threshold levels (5 pmol/l, (1), 22 pmol/l, (7)) were used for comparison.

β-Hydroxybutyrate levels were measured by an automated kinetic method in samples collected in vacutainer tubes containing sodium fluoride and potassium oxalate (13).

Calculation of insulin/blood glucose, proinsulin/insulin and proinsulin/blood glucose indices

The ratio of insulin to blood glucose was calculated as follows: insulin (mIU/l)/blood glucose (g/l). Turner's

ratio was calculated as follows: insulin (mIU/l)/(blood glucose (mg/dl)-30)×100. The ratio of proinsulin to insulin was calculated as proinsulin (pmol/l)/insulin (mIU/l) and the ratio of proinsulin to blood glucose as proinsulin (pmol/l)/blood glucose (g/l).

The thresholds used in our study as references for comparison were 30 for insulin/blood glucose ratio and 50 for Turner's ratio.

Statistical analysis

Non-parametric methods such as the Mann–Whitney *U* test were used for statistical analysis (Statview 5 program, SAS Institute Inc., Cary, NC, USA). The results were considered to be significant if $P<0.05$.

Receiver-operating characteristic (ROC) curves were constructed using the NCSS 2000 program (Kaysville, UT, USA) to examine the diagnostic test performance. Sensitivity against 1-specificity was plotted at each threshold level and the area under the curve (AUC) was computed by the non-parametric Wilcoxon test. AUC represents the probability of correctly identifying controls and patients with endogenous hyperinsulinism. A value of 0.5 means that the result is no better than chance.

Results

Blood glucose, insulin, C-peptide and proinsulin after an overnight fast

The patients with endogenous hyperinsulinism had lower blood glucose, higher proinsulin, insulin and C-peptide levels than controls (Table 1). Out of 33, 20 (61%) patients had blood glucose levels between 2.5 and 3.3 mmol/l and 9 patients (27%) had levels below 2.5 mmol/l, whereas all controls had levels above 3.3 mmol/l. Since the diagnosis of endogenous hyperinsulinism is easily made by measuring C-peptide or proinsulin in patients with morning blood glucose levels below 2.5 mmol/l (1), we selected only those 24 out of 33 patients with morning blood glucose levels above 2.5 mmol/l and compared their results with those of the 67 controls in order to determine if proinsulin, insulin or C-peptide levels after an overnight fast could make the diagnosis of insulinoma even when blood glucose levels do not decrease below 2.5 mmol/l.

Table 2 shows the sensitivity and specificity observed with the various parameters studied using the cut-off values previously established or determined with the ROC curves after an overnight fast. Proinsulin levels appear to be the best and most simply evaluated parameter to distinguish patients from controls, either with the already established cut-off value of 22 pmol/l or with the 20.7 pmol/l cut-off value established with the ROC curves. The sensitivity and specificity obtained with the proinsulin/blood glucose and the proinsulin/insulin-IRMA ratios with the cut-off values determined using the ROC curves are similar to those of proinsulin levels. Insulin, C-peptide levels, Turner's ratio or insulin/blood glucose ratio did not improve the diagnostic accuracy with the cut-off values previously reported and those established with the ROC curves (data not shown).

Proinsulin levels were above 22 pmol/l in 24 out of 33 patients (73%), whereas they were below 22 pmol/l in 66 out of 67 controls (sensitivity = 73%, specificity = 98%). The only control with a proinsulin level above 22 pmol/l was obese (BMI = 31 kg/m^2). Considering the non-obese subjects, proinsulin levels were above 22 pmol/l in 73% of the patients and in none of the controls. Thus, in non-obese patients and controls (BMI < 30 kg/m^2), the specificity of proinsulin after an overnight fast with a threshold of 22 pmol/l reached 100% and was better than in the entire population, while the sensitivity was similar (=70%). Sensitivity and specificity in non-obese patients and controls were not improved by the cut-off value of 20.7 pmol/l established with the ROC curves (data not shown).

Blood glucose levels during the fast test

The fast test lasted 8±11 h (0–48 h) in the patients and 72 h in all the controls. At the end of the fast test, blood glucose levels were 2.0±0.3 mmol/l (1.2–2.4 mmol/l) in patients, whereas they were 4.5±0.5 mmol/l (3.5–6.0 mmol/l) in controls ($P<0.0001$). All the patients and 4 controls reached blood glucose levels below 2.5 mmol/l. The controls who reached such blood glucose levels (2.3–2.4 mmol/l) were four women aged 52±23 years (32–85 years) with a BMI of 19.8±2.3 kg/m^2 (17.4–22.3 kg/m^2).

Table 1 Blood glucose, insulin, C-peptide and proinsulin levels in 33 patients and in 67 controls after an overnight fast. To convert insulin values to mIU/l, divide by 7.175. To convert C-peptide values to ng/ml, multiply by 3.

	Patients		Controls		
	Mean±S.D.	Range	Mean±S.D.	Range	*P*
Blood glucose (mmol/l)	3.0±1.0	1.3–5.1	4.5±0.5	3.5–6.0	<0.0001
Proinsulin (pmol/l)	79.2±100.6	3.3–460	8.4±4.9	2–31	<0.0001
Insulin (pmol/l)	81.1±58.8	14.3–265.5	45.9±27.2	7.2–143.5	<0.004
C-peptide (nmol/l)	0.8±0.4	0.2–2	0.5±0.2	0.03–1	<0.0002

Table 2 Sensitivity and specificity of hormonal parameters established with previous thresholds or with receiver-operating characteristic (ROC) curves and evaluated after an overnight fast in patients and controls with fasting blood glucose level >2.5 mmol/l. The proinsulin threshold of 22 pmol/l, the insulin threshold of 21 pmol/l (3 mUI/l), the C-peptide threshold of 0.2 nmol/l (0.6 ng/ml), the insulin/blood glucose threshold of 30, the Turner's ratio threshold of 50 were the previously established thresholds (1, 3, 7); notice that the 22 pmol/l proinsulin threshold is the only one that has been reported to identify the patients with endogenous hyperinsulinism whatever the concomitant blood glucose level may be (7). The other thresholds were established using ROC curves in 23 patients with blood glucose levels after an overnight fast above 2.5 mmol/l and in 67 controls. For calculation of the ratios, insulin was expressed in mIU/l, blood glucose levels in g/l and proinsulin in pmol/l (see Methods).

	Threshold value	Sensitivity (%)	Specificity (%)
Insulin-IRMA	21 pmol/l	67	19
Insulin-ICL	21 pmol/l	90	10
C-peptide	0.2 nmol/l	100	12
Proinsulin	22 pmol/l	73	98
	20.7 pmol/l	76	97
Insulin-IRMA/blood glucose	30	100	0
	11.4	29	100
Insulin-ICL/blood glucose	30	100	0
	11	70	86
Turner's ratio (with insulin-IRMA)	50	29	100
	20.7	64	95
Turner's ratio (with insulin-ICL)	50	5	100
	21.8	59	90
Proinsulin/blood glucose	15.4	91	87
Proinsulin/insulin-IRMA	3.8	83	90
Proinsulin/insulin-ICL	2.9	56	93

Diagnostic accuracy of proinsulin, insulin and C-peptide levels and derived ratios when blood glucose levels were 2.5–3.3 mmol/l during the 72-h fast test

Thirty-one patients and 47 controls had blood glucose levels between 2.5 and 3.3 mmol/l during the fast test. Such blood glucose levels were reached after 7 ± 10 h of fasting (0–34 h) in patients versus 47 ± 17 h (24–72 h) in controls.

Table 3 shows the sensitivity and specificity of the various parameters studied using the cut-off values previously established or determined with the ROC curves. Proinsulin levels appear to be the best parameter, either with the already established cut-off value of 22 pmol/l or with the 24 pmol/l cut-off value established with the ROC curves (specificity = 100%, sensitivity = 73–76%).

Proinsulin levels were higher in the 31 patients who presented with blood glucose levels between 2.5 and 3.3 mmol/l during the fast test than in the 47 controls with similar blood glucose levels (Table 4). Proinsulin levels were below 5 pmol/l in 22 controls (47%), in the patient with nesidioblastosis and in none of the insulinoma patients. On the other hand, proinsulin levels were above 22 pmol/l in 23 (74%) insulinoma patients and in none of the controls.

Neither insulin nor C-peptide levels can be used to make or to rule out the diagnosis of inappropriate insulin secretion when blood glucose is 2.5–3.3 mmol/l (Tables 5 and 6). However, with the cut-off values established by the ROC curves (31.6 pmol/l for insulin-IRMA and 28 pmol/l for insulin-ICL), diagnostic specificity reaches 89–95%.

Insulin-IRMA or Insulin-ICL and C-peptide levels were higher ($P < 0.0001$) in patients than in controls (Table 4). However, there was an overlap between the results observed in patients and those of the controls when blood glucose levels reached 2.5–3.3 mmol/l during the fast test.

The ratios evaluated in the present study had either poor sensitivity or specificity in comparison with those of proinsulin levels, except the proinsulin/blood glucose ratio, which gave sensitivity and specificity almost similar to those of proinsulin levels (Table 3).

Diagnostic accuracy of proinsulin, insulin and C-peptide levels and derived ratios during the 72-h fast test at the time when blood glucose levels are below 2.5 mmol/l

Proinsulin levels were above the 5 pmol/l threshold in all patients, below the 22 pmol/l threshold in 7 out of 33 patients (22%) and below 5 pmol/l in the four controls who reached blood glucose levels below 2.5 mmol/l at the end of the fast (Tables 5 and 6).

Insulin-IRMA levels were below 21 pmol/l in 6 out of the 17 (35.3%) patients and insulin-ICL levels were below 21 pmol/l in 3 out of the 28 (11%) patients. In the four controls with blood glucose level below 2.5 mmol/l, insulin-ICL levels were low.

C-peptide levels were above 0.2 nmol/l in all insulinoma patients. The only patient who had C-peptide levels below 0.2 nmol/l (=0.1 nmol/l) was the patient with nesidioblastosis. The four controls who reached blood glucose levels below 2.5 mmol/l had concomitant C-peptide levels below 0.2 nmol/l.

Regarding the traditional ratios calculated with insulin levels, their diagnostic accuracy was lower than that of proinsulin or C-peptide levels (data not shown). If the thresholds established with the ROC curves for blood glucose levels of 2.5–3.3 mmol/l were used for the results observed when blood glucose levels were below 2.5 mmol/l, the diagnostic accuracy of these ratios improved but remained poor. The insulin-IRMA/blood glucose ratio still did not reach the threshold of 11.4 in 53% of the patients (9 out of 17 patients) and the same ratio calculated with insulin-ICL did not reach the threshold of 7.3 in 11% of the patients (3 out of 28 patients). Turner's ratio calculated with

Table 3 Sensitivity and specificity of hormonal parameters established with previous thresholds or with receiver-operating characteristic (ROC) curves and evaluated in patients and controls at the time of blood glucose between 2.5 and 3.3 mmol/l. The proinsulin thresholds of 5 and 22 pmol/l, the insulin threshold of 21 pmol/l (3 mIU/l), the C-peptide threshold of 0.2 nmol/l (0.6 ng/ml), the insulin/blood glucose threshold of 0.3, and the Turner's ratio threshold of 50 were the previously established thresholds (1, 3, 7). The other thresholds were established using ROC curves in 31 patients and in 47 controls with blood glucose levels between 2.5 and 3.3 mmol/l. For calculation of the ratios, insulin was expressed in mIU/l, blood glucose levels in g/l and proinsulin in pmol/l (see Methods).

	Threshold	Sensitivity (%)	Specificity (%)
Insulin-IRMA	21 pmol/l (3 mIU/l)	56	50
	31.6 pmol/l (4.4 mUI/l)	41	95
Insulin-ICL	21 pmol/l (3 mUI/l)	81	45
	28 pmol/l (3.9 mIU/l)	76	89
C-peptide	0.20 nmol/l (0.6 ng/ml)	90	55
	0.23 nmol/l (0.7 ng/ml)	90	84
Proinsulin	5 pmol/l	97	42
	22 pmol/l	76	100
	24 pmol/l	73	100
Insulin-IRMA/blood glucose	30	100	0
	11.4	29	100
Insulin-ICL/blood glucose	30	100	2
	7.3	71	92
Turner's ratio	50	25	100
(with insulin-IRMA)	21	41	95
Turner's ratio	50	33	100
(with insulin-ICL)	16	76	92
Proinsulin/blood glucose	26.8	77	97
Proinsulin/insulin-IRMA	7.8	47	95

insulin-IRMA did not reach the threshold of 21 in 6 out of 16 (37.5%) patients and the same index calculated with insulin-ICL remained below the threshold of 16 in 3 out of 28 (11%) patients.

Regarding the ratios calculated from proinsulin levels, if the thresholds established with the ROC curves at the time when blood glucose levels were between 2.5 and 3.3 mmol/l were used for the values observed with concomitant blood glucose levels below 2.5 mmol/l, the four controls had normal results but proinsulin/insulin-IRMA ratio was below 7.8 in 9 out of 17 (53%) patients, proinsulin/insulin-ICL ratio was below 7.2 in 12 out of 28 (43%) patients and proinsulin/blood glucose ratio was below 26.8 in 3 out of 33 (9%) patients.

Diagnostic accuracy of proinsulin during the 72-h fast test at the time when blood glucose levels are below 2 mmol/l

Since no control subject reached blood glucose levels below 2 mmol/l during the fast test, we studied the values found in those 21 insulinoma patients in whom the fast test was prolonged until they experienced symptomatic hypoglycaemia below 2 mmol/l (1.8 ± 0.2 mmol/l, range 1.2–2 mmol/l). At the time of such blood glucose levels, proinsulin levels above 5 pmol/l and C-peptide levels above 0.2 nmol/l were still the best parameters to identify an inappropriate insulin secretion, while insulin levels or insulin/glucose ratio were below the diagnostic thresholds in some patients. Proinsulin levels were 95.3 ± 89.8 pmol/l (range 7.4–425), insulin-ICL levels were 90.3 ± 56.8 pmol/l (range 7.2–186.6), insulin-IRMA levels were 91.5 ± 99.8 pmol/l (range 7.2–301.4) and C-peptide levels were 0.9 ± 0.5 nmol/l (range 0.4–2.6).

Discussion

The biological diagnosis criteria of endogenous hyperinsulinism have been challenged within the last years. In the past years, ratios calculated with insulin, blood glucose levels, then with proinsulin and insulin levels had been recommended (1, 3, 7). New assays leading to sensitive and specific evaluation of insulin, C-peptide and proinsulin are now in use and several thresholds were determined in order to make the diagnosis of endogenous hyperinsulinism. At the time of hypoglycaemia below 2.5 mmol/l, threshold levels of 0.2 nmol/l for C-peptide and 3 mIU/l (21 pmol/l) for insulin (with an insulin-specific immunochemiluminometric assay) were recommended by Service (3); the proinsulin threshold was found to be 5 pmol/l for such

Table 4 Insulin, C-peptide and proinsulin levels in 31 patients and in 47 controls when blood glucose level was between 2.5 and 3.3 mmol/l during the 72-h fast.

	Patients		Controls		
	Mean ± S.D.	Range	Mean ± S.D.	Range	*P*
Proinsulin (pmol/l)	90 ± 115	2.8–540	5.9 ± 3.6	2–21	< 0.0001
Insulin-IRMA (pmol/l)	54.5 ± 63.8	3.2–208.1	15.8 ± 9.3	2.1–46.6	< 0.0009
Insulin-ICL (pmol/l)	63.1 ± 58.8	7.2–344.4	15.8 ± 10.0	0.7–64.1	< 0.0001
C-peptide (nmol/l)	0.7 ± 0.5	0.07–2	0.17 ± 0.1	0.03–0.6	< 0.0001

Table 5 Insulin, C-peptide and proinsulin levels in 33 patients and in 4 controls when blood glucose level was below 2.5 mmol/l. In all patients, we collected at least two samples with blood glucose, insulin, C-peptide and proinsulin levels at the time of such blood glucose levels.

	Patients		Controls	
	Mean ± S.D.	Range	Mean ± S.D.	Range
Proinsulin (pmol/l)	113 ± 140	7–720	2.3 ± 0.6	2–3.2
Insulin-IRMA (pmol/l)	66 ± 76	5–344	ND	ND
Insulin-ICL (pmol/l)	70 ± 47	7–186	12.5 ± 3.6	7–14.2
C-peptide (nmol/l)	0.8 ± 0.5	0.1–2.6	0.1 ± 0.03	0.07–0.13

blood glucose levels (1), while others suggested a 22 pmol/l threshold (7). Others recommended different cut-off levels (2, 7).

In the present study, we confirm our previous finding that at the time of hypoglycaemia below 2.5 mmol/l, several patients (11–35%) with endogenous hyperinsulinism have insulin levels below 21 pmol/l (4). Using two insulin-specific assays, both devoid of any significant cross-reaction with intact proinsulin, 35% of the patients with endogenous hyperinsulinism had an insulin-IRMA level below 21 pmol/l and 11% of them had an insulin-ICL level below 21 pmol/l. Thus, at the time of symptomatic hypoglycaemia below 2.5 mmol/l, an insulin level below 21 pmol/l cannot rule out the diagnosis of endogenous hyperinsulinism (4–6).

On the other hand, a concomitant serum C-peptide level above a 0.2 nmol/l cut-off value is an excellent criterion to establish the diagnosis of insulinoma. In our study, serum C-peptide was above 0.2 nmol/l in all insulinomas. The only patient with a C-peptide level below 0.2 nmol/l, at the time of hypoglycaemia below 2.5 mmol/l was the patient with nesidioblastosis. In this patient, such results were observed when hypoglycaemia reappeared 1 year after left-sided pancreatectomy. The lower secretion of glucagon consecutive to the partial pancreatectomy might at least partly explain

Table 6 Data at discontinuation of fasts in 33 patients with endogenous hyperinsulinism. All patients were symptomatic at the time of discontinuation of the fast test. Duration of fast = 0 indicates that the patient had spontaneous symptomatic hypoglycaemia at the beginning of their stay in the hospital for the fast test and as of medical supervision of the fast was started; in such patients, blood samples were collected twice within the first 20–30 min of the test and then the fast was discontinued.

Patients	Duration of fast	Blood glucose (mmol/l)	Insulin levels (pmol/l)	C-peptide levels (nmol/l)	Proinsulin levels (pmol/l)	Insulin (mIU/l/blood glucose g/l)	Turner's ratio
1	10	2.09	57.4[a]	0.9	400	21.1	100
2	1	2.47	100.5[a]	1.1	60	31.1	93.3
3	4	2.31	21.5[a]	0.4	20	7.1	25
4	0	2.42	31.6[a]	0.4	14	10	31.4
5	4	1.82	208.1[b]	1.8	37	65.8	312.5
6	30	1.93	14.4[b]	0.4	24	5.7	40
7	2	1.98	93.3[a]	1.0	425	36.1	216.7
8	4	2.15	86.1[a]	1.4	35	30.8	133.3
9	1	2.37	122[b]	0.9	11	39.5	130.8
10	4	2.37	7.2[b]	0.4	34	2.3	7.7
11	4	1.27	301.4[b]	2.6	96	182.6	25.8
12	1	2.15	35.9[a]	0.5	16	12.8	55.6
13	1	1.21	150.7[a]	1.3	198	95.5	1300.0
14	1	1.93	14.4[a]	0.7	82	5.71	40
15	4	1.93	43.1[a]	0.6	71	17.1	120
16	1	2.15	93.3[a]	1.0	111	31	108.3
17	10	1.87	157.9[a]	1.2	13	64.7	550
18	48	2.20	7.2[a]	0.1	7	2.5	10
19	20	2.09	107.6[a]	0.7	132	39.5	187.5
20	4	2.26	93.3[a]	0.8	550	31.7	118.2
21	24	2.47	64.6[a]	0.9	240	20	60
22	24	1.98	157.9[a]	0.6	37	61.1	366.7
23	4	1.98	35.9[a]	0.3	210	15.6	250
24	24	1.92	165[a]	1.4	71	67.6	575.0
25	1	2.14	35.9[a]	0.4	59	12.8	55.6
26	4	2.42	35.9[a]	0.3	14	11.4	35.7
27	0	1.98	93.3[a]	0.7	160	36.1	216.7
28	10	1.87	57.4[a]	0.9	96.8	18.2	57.1
29	0	1.70	122[a]	1.0	73	54.9	1700
30	4	1.98	43.1[a]	0.6	7.4	16.7	100
31	0	2.03	57.4[a]	0.9	92	9.1	28.6
32	10	1.80	35.9[a]	0.5	82	15.2	166.7
33	8	2.42	7.2[a]	0.3	25	2.27	7.1

[a]ICL insulin.
[b]IRMA insulin.

that symptomatic hypoglycaemia could occur in this patient with lower insulin and C-peptide levels than those of the insulinoma patients. In addition, it has already been observed that patients with nesidioblastosis can present a lower secretion of C-peptide and insulin than insulinoma patients (14, 15), so that the diagnosis of nesidioblastosis is often more difficult than that of insulinomas. Moreover, it has been shown that patients with nesidioblastosis and even some rare glucose-sensitive insulinoma patients can undergo a 72-h fast test without hypoglycaemia (15), such patients being detected only with an oral glucose tolerance test (OGTT) or a meal test.

In our study, proinsulin levels above 5 pmol/l during the fast test at the time of hypoglycaemia below 2.5 mmol/l appears to be the best criterion to make the diagnosis of endogenous hyperinsulinism with both 100% sensitivity and specificity. The four controls that experienced blood glucose levels below 2.5 mmol/l had concomitant proinsulin below 5 pmol/l. Conversely, all the patients with endogenous hyperinsulinism had proinsulin levels above 5 pmol/l but 18% of them had proinsulin below 22 pmol/l. The 5 pmol/l, not the 22 pmol/l, also identifies all the insulinoma patients who reach blood glucose levels not reached by any of the controls, i.e. levels below 2 mmol/l. Therefore, a proinsulin level above 5 pmol/l is an excellent criterion to make the diagnosis of endogenous hyperinsulinism. On the contrary, the 22 pmol/l threshold would lead to several false negative diagnoses and it cannot be recommended as a diagnostic criterion at the time of hypoglycaemia below 2.5 mmol/l.

Traditional blood glucose/insulin ratio and Turner's ratio cannot differentiate accurately patients with endogenous hyperinsulinism and controls, either with the thresholds previously recommended or with the new threshold values established in our study with ROC curves. In the same way, the ratio of blood glucose levels to proinsulin and that of proinsulin level to insulin level did not result in a better diagnostic accuracy than proinsulin levels themselves at the time of hypoglycaemia below 2.5 mmol/l. Therefore, our results confirm that these ratios should not be used at present.

In our study, all patients with endogenous hyperinsulinism experienced hypoglycaemia below 2.5 mmol/l either spontaneously or during the fast test. However, it has been reported that some rare insulinoma patients might not reach blood glucose levels below 2.5 mmol/l even after 72-h fast (16–21). In addition, the 72-h fast test is not well tolerated in all subjects. Thus, it would have been interesting to have biochemical diagnostic criteria allowing the fast test to be shortened or even to avoid it in patients with endogenous hyperinsulinism who do not present spontaneously with symptomatic hypoglycaemia below 2.5 mmol/l, while also ruling out the diagnosis in subjects without endogenous hyperinsulinism.

First, we confirm that healthy subjects can have blood glucose levels below 3.3 mmol/l or even below 2.5 mmol/l during the fast test (1, 22), the lowest level being above 2 mmol/l (2.3 mmol/l) in our study.

When blood glucose levels are between 2.5 and 3.3 mmol/l during the fast test, insulin and C-peptide levels overlapped between controls and patients (9). Thus, they cannot be used as diagnostic tools even when measured with new specific assays. For such blood glucose levels, it is known that β-hydroxybutyrate levels represent the most accurate diagnostic criterion. In our study, at the time of such blood glucose levels, proinsulin levels above 22 pmol/l would make the diagnosis of endogenous hyperinsulinism in part of the patients. Conversely, proinsulin levels below 5 pmol/l would rule out the diagnosis of insulinoma in our patients, not the diagnosis of nesidioblastosis. However, no conclusion can be drawn for subjects whose proinsulin levels are between 5 and 22 pmol/l for such blood glucose levels. The use of ROC curves did not allow to establish a new proinsulin threshold improving the diagnostic accuracy of proinsulin compared with the thresholds previously suggested. The diagnostic accuracy of the ratios calculated from insulin and proinsulin levels is not better than that of proinsulin itself, either with the previously cut-off values or with the new thresholds established with the ROC curves.

The fast test is a long and painful test requiring hospitalization for a few days. It would have been interesting to find either a screening test that is easy to carry-off in outpatients and could permit to select all the patients with endogenous hyperinsulinism, or a diagnostic test that could make the diagnosis of endogenous hyperinsulinism without performing the fast test in patients who do not present with spontaneous symptomatic hypoglycaemia below 2.5 mmol/l.

Proinsulin levels after an overnight fast could be of interest in the screening of patients with endogenous hyperinsulinism (7). In our study, its sensitivity and specificity (with a threshold of 22 pmol/l) were 73 and 98% respectively. All controls had proinsulin levels below 22 pmol/l except an obese subject. Therefore, proinsulin levels above 22 pmol/l would have made the diagnosis of endogenous hyperinsulinism with a 100% specificity in our non-obese subjects, so that the fast test would not have been necessary in 73% of the non-obese patients with endogenous hyperinsulinism. On the other hand, based on our study, it must be pointed out that morning proinsulin levels below 22 pmol/l do not rule out the diagnosis of endogenous hyperinsulinism. The ROC curves did not enable us to determine a new threshold value that improved the diagnostic accuracy of proinsulin levels after an overnight fast. In obese subjects, after an overnight fast, proinsulin levels above 22 pmol/l do not make the diagnosis of endogenous hyperinsulinism: in 110 obese subjects who did not present with glycaemic disorders (blood

glucose levels below 6.05 mmol/l (1.10 g/l)) or hypoglycaemia related to endogenous hyperinsulinism), proinsulin level was 16.8 ± 11.4 pmol/l (3.5–78 pmol/l) and 23 of these (21%) had proinsulin levels above 22 pmol/l after an overnight fast (unpublished data). Other factors can contribute to insulin resistance and increased insulin and proinsulin levels; and such factors might be overlooked or unknown in individual patients, so that one must be very cautious with the use of proinsulin levels after an overnight fast for the diagnosis of insulinoma patients. Based on our study, a morning proinsulin level above 22 pmol/l in a non-obese, non-insulin-resistant subject should lead to high suspicion of insulinoma, but whether this can be used as a diagnostic criterion cannot be assessed and would need further study in a large cohort of subjects.

Conclusion

In conclusion, based on our data, among the parameters evaluated, the most reliable method to make or to rule out the diagnosis of fasting hypoglycaemia related to endogenous hyperinsulinism is to use a 5 pmol/l serum proinsulin threshold at the time of hypoglycaemia below 2.5 mmol/l (0.45 g/l) during a fast test. For such blood glucose levels, all insulinoma patients and the nesidioblastosis patient had proinsulin levels exceeding 5 pmol/l, while none of the control subjects reached such proinsulin levels with similar blood glucose levels. No ratio calculated with proinsulin, insulin or blood glucose levels gave a better diagnostic accuracy. C-peptide levels with a 0.2 nmol/l (0.6 ng/ml) minimum threshold at the time of hypoglycaemia below 2.5 mmol/l (0.45 g/l) also made the correct diagnosis in all our insulinoma patients and missed the diagnosis only in the patient with nesidioblastosis. We confirm that the diagnostic accuracy of insulin levels is much less than that of C-peptide and proinsulin, and that insulin levels below 3 mIU/l (21 pmol/l) concomitant with hypoglycaemia below 2.5 mmol/l (0.45 g/l) cannot rule out the diagnosis of endogenous hyperinsulinism, when insulin is measured with the insulin-specific assays now in use.

Proinsulin levels with a cut-off value of 22 pmol/l (not insulin, C-peptide levels or ratios calculated with insulin and blood glucose levels) are partly of interest for the diagnosis of insulinoma when measured in patients with blood glucose of 2.5–3.3 mmol/l during the fast test or after an overnight fast in non-obese subjects, but their diagnostic accuracy is much less than that found with the 5 pmol/l threshold when concomitant blood glucose levels are below 2.5 mmol/l. No basal data, including normal proinsulin levels after an overnight fast, can rule out the diagnosis of endogenous hyperinsulinism, so the fast test (if necessary combined with an OGTT, especially when nesidioblastosis is suspected) is and remains the gold standard for the diagnosis of endogenous hyperinsulinism.

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