Comparative value of plasma ACTH and beta-endorphin measurement with three different commercial kits for the etiological diagnosis of ACTH-dependent Cushing’s syndrome

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Recent reports suggest that, contrary to radioimmunoassays (RIA), immunoradiometric assays (IRMA) artifactually decrease plasma ACTH levels in patients with the ectopic ACTH syndrome. Discrepancies between RIA and IRMA results may provide a means of discriminating this entity from Cushing’s disease. We have compared the results of these two techniques, together with those of a beta-endorphin assay, in 17 patients with Cushing’s disease, 9 with the ectopic ACTH syndrome and 30 controls. ACTH-RIA and ACTH-IRMA levels in patients with Cushing’s disease were similar (17.5 ± 2.5 vs 15.1 ± 2.8 pmol/l) and were correlated (r = 0.59, p < 0.01). ACTH-RIA levels in patients with the ectopic ACTH syndrome were higher than ACTH-IRMA levels (27.3 ± 2.9 vs 14.5 ± 2.5, p < 0.01) and these did not correlate. The ACTH-RIA and ACTH-RIA/ACTH-IRMA ratio levels in patients with the ectopic ACTH syndrome were higher than those of patients with Cushing’s disease (p < 0.01), but they overlapped with these in 27 and 31% of cases respectively. Plasma beta-endorphin level was higher in patients with the ectopic ACTH syndrome than in patients with Cushing’s disease (81.9 ± 19.4 vs 26.4 ± 5.6 pmol/l, p < 0.01) and was correlated with ACTH only in patients with Cushing’s disease. The overlap in beta-endorphin and beta-endorphin/ACTH-IRMA molar ratio levels between the two groups were 19 and 27%, respectively. Although no parameter could be used to make clearcut distinction between Cushing’s disease and the ectopic ACTH syndrome, the discriminative power of beta-endorphin level was clearly better than that of the comparison between ACTH-RIA and ACTH-IRMA levels.

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The etiologies of Cushing’s syndrome can be classified as ACTH-dependent causes which include Cushing’s disease and the ectopic ACTH syndrome, and as ACTH-independent causes such as primary adrenocortical diseases (1). The distinction between these two entities is the first step of the etiological investigation of Cushing’s syndrome. It is usually resolved with plasma ACTH measurement (2). The poor sensitivity of most of the commercially available radioimmunoassays for ACTH (ACTH-RIA) do not provide reliable discrimination between ACTH-dependent and independent entities. However, the sensitivity of the assay has recently been improved by the development of two-site immunoradiometric assays (ACTH-IRMA) (3, 4). On the contrary, it still remains difficult to distinguish between Cushing’s disease and the ectopic ACTH syndrome (1, 5). In this etiological investigation, plasma ACTH levels are poorly discriminative (5) but the presence of ACTH fragments (6–8) or large molecular forms (“Big” ACTH) is highly suggestive of the ectopic ACTH syndrome (7, 9–11). Demonstration of such abnormalities requires chromatographic characterization of ACTH immunoreactivity, which is time-consuming, or the use of specific assays for ACTH precursors which are not commercially available (12). The high specificity of ACTH-IRMA does not allow the detection of “Big ACTH” or ACTH fragments which can artifactually decrease the ACTH 1–39 concentration (4). Conversely, some of these ACTH variants can be detected with ACTH-RIA. Thus, some authors have pointed out that discrepancies between ACTH-RIA and IRMA results are suggestive of the ectopic ACTH syndrome (4).

Elsewhere, the measurement of other proopio-melanocortin end-product peptides such as beta-lipotropin (beta-LPH), has proven to be useful for the differential diagnosis between Cushing’s disease and ectopic ACTH syndrome (13–14). Few reports suggest that plasma or tissue beta-endorphin concentrations in the ectopic ACTH syndrome may differ from those observed in Cushing’s disease (10, 15–18). However, no assay for beta-endorphin measurement in clinical practice has been available until recently.

The aim of the present study was to evaluate the usefulness of the simultaneous measurements of ACTH-
RIA, ACTH-IRMA and β-endorphin in a single basal plasma specimen, with commercially available kits for the diagnosis of ACTH-dependent Cushing’s syndrome.

Patients and methods

Twenty-six patients with ACTH-dependent Cushing’s syndrome were studied. All of them presented the typical clinical features of the syndrome.

Endocrine evaluation of all patients included CRH and dexamethasone tests which were performed as previously described (19).

Seventeen patients (13F and 4M, age ± SD: 46.4 ± 13.7 years) had histologically proven Cushing’s disease and/or were cured after transsphenoidal pituitary microsurgery (2 cases of macroadenoma, 13 cases of microadenoma, 2 cases of diffuse corticotroph hyperplasia). Nine patients had an ectopic ACTH syndrome (5F and 4M, age ± SD: 47.2 ± 19.3 years). Six of them had an histologically proven ectopic ACTH secreting tumour (two cases of thymic tumour, two cases of lung carcinoma, one case of pancreatic tumour and one case of medullary thyroid carcinoma). In three patients, no tumour was detectable by computerized tomographic (CT) scanning of the neck, the whole lung and the abdomen, nor by nuclear magnetic resonance imaging of the pituitary. The existence of an occult ectopic ACTH-secreting tumour was suspected, as previously described (20), on the basis of a low central to peripheral ACTH gradient during inferior petrosal sinus sampling combined with CRH injection (< 1.3).

Thirty healthy subjects served as controls.

Assays

Blood samples were taken at 08.00 in all patients and in 18 healthy controls. In addition, blood was sampled from 12 healthy controls at 00.00. Samples were collected in EDTA-containing tubes and were immediately centrifuged. After separation, plasma was frozen at −80°C until assay.

ACTH-IRMA was measured with the Allegro HS-ACTH kit from Nichols Institute (San Juan Capistrano, USA). The least detectable concentration was 0.5 pmol/l. The intra- and interassay coefficients of variation were below 6 and 8% respectively. ACTH-RIA was measured with the ACTH K-PR kit from Oris Industries (Gif-sur-Yvette, France). The least detectable concentration was 4.4 pmol/l. The intra- and interassay coefficients of variation were below 7.5 and 10% respectively. β-endorphin was measured with the Allegro IRMA kit from the Nichols Institute. The least detectable concentration was 3 pmol/l. The intra- and interassay coefficients of variation were below 6 and 10% respectively. Plasma cortisol was also measured by RIA (IM-Cort. Immunotech, Marseille, France). The intra- and interassay coefficients of variation were below 5 and 7% respectively.

All plasma samples were simultaneously assayed with the four hormonal kits.

Statistics

All results are expressed as the mean ± SEM. In the case of undetectable concentration a value equal to the least detectable concentration of the assay was assigned for statistical calculations. Comparisons between groups were performed by ANOVA followed by Fisher Protected Least Significant Difference as post test. Comparisons within groups were performed with the use of Wilcoxon’s test for paired values. Linear regression analysis was done with the use of Spearman’s rank test.

Individual responses criteria

For the examination of individual results, a value was considered to be consistent with the diagnosis of the ectopic ACTH syndrome if it was above the mean + 1 so observed in patients with Cushing’s disease. The overlap between the two groups was calculated as the number of patients with Cushing’s disease exhibiting values above this threshold added to the number of patients with ectopic ACTH syndrome exhibiting values below this threshold divided by the total number of subjects studied.

Ethics

The procedures followed were in accordance with the ethical standards of the institutionally responsible committee on human experimentation.

Results

Controls (Table 1)

Plasma ACTH at 08.00 was below the least detectable concentration in 10 of 18 subjects using the RIA while measurable concentrations were obtained in all using the IRMA. β-endorphin was detectable in all subjects at 08.00 and was correlated with ACTH-IRMA (r = 0.58; p < 0.05) but not with ACTH-RIA concentration (r = 0.47).

Plasma ACTH in samples obtained at 00.00 was detectable in only 1 of 12 subjects (6.4 pmol/l) with RIA and in 9 with IRMA (2.7 ± 0.5 pmol/l; range: 1.6–6.4). β-endorphin was detectable in 5 of 12 subjects (5.0 ± 0.9 pmol/l; range: 3.1–7.5 pmol/l).

Patients with Cushing’s disease (Table 1)

Plasma ACTH was detectable in all patients with both RIA and IRMA. The ACTH-RIA concentration was correlated with the ACTH-IRMA concentration
Table 1. Results of the endocrine investigation (mean ± SEM) performed in a 08.00 plasma sample in 17 patients with Cushing’s disease (CD), 9 patients with the ectopic ACTH syndrome (EAS) and 18 healthy control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CD</th>
<th>EAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (nmol/l)</td>
<td>525.7±43.4</td>
<td>630.9±51.9</td>
<td>1046.2±201.3**</td>
</tr>
<tr>
<td>ACTH-RIA (pmol/l)</td>
<td>5.6±0.5</td>
<td>17.5±2.5*</td>
<td>27.3±2.9**</td>
</tr>
<tr>
<td>ACTH-IRMA (pmol/l)</td>
<td>6.0±0.8</td>
<td>15.1±2.8*</td>
<td>14.5±2.5*</td>
</tr>
<tr>
<td>ACTH-RIA/ACTH-IRMA ratio</td>
<td>1.2±0.2</td>
<td>1.5±0.3</td>
<td>2.5±0.5†</td>
</tr>
<tr>
<td>β-endorphin (pmol/l)</td>
<td>7.8±0.9</td>
<td>26.4±5.6</td>
<td>81.9±19.4**</td>
</tr>
<tr>
<td>β-endorphin/ACTH-IRMA ratio</td>
<td>1.4±0.2</td>
<td>2.3±0.5</td>
<td>7.0±2.1**</td>
</tr>
<tr>
<td>β-endorphin/ACTH-RIA ratio</td>
<td>1.4±0.2</td>
<td>1.6±0.2</td>
<td>3.0±0.6**</td>
</tr>
</tbody>
</table>

‡: p<0.05 in comparison with control subjects. †: p<0.01 in comparison with control subjects. †: p<0.05 in comparison with CD patients.

*: p<0.01 in comparison with CD patients.

(r_s=0.59; p<0.02) (Fig. 1A). The ACTH-RIA/ACTH-IRMA ratio did not differ from that of controls.

β-endorphin concentration was measurable in all patients and correlated with ACTH-RIA (r_s=0.78; p<0.001) and with ACTH-IRMA concentrations (r_s=0.68; p<0.01) (Fig. 2A). The β-endorphin/ACTH-IRMA ratio and the β-endorphin/ACTH-RIA ratio were not different from those of controls.

Patients with EAS (Table 1)

ACTH-RIA concentrations in patients with the ectopic ACTH syndrome were higher than ACTH-IRMA concentrations. ACTH-RIA concentrations differed from those of Cushing’s disease patients while ACTH-IRMA concentrations were similar. There was no correlation between ACTH-RIA and ACTH-IRMA (r_s=0.46; p>0.2) (Fig. 1B). The ACTH-RIA/ACTH-IRMA ratio was higher than in patients with Cushing’s disease and in controls.

β-endorphin concentrations were higher than in patients with Cushing’s disease and did not correlate with ACTH-RIA (r_s=0.45; p>0.2) nor with ACTH-IRMA concentrations (r_s=0.22; p>0.5) (Fig. 2B). The β-endorphin/ACTH-RIA and β-endorphin/ACTH-IRMA molar ratio were higher than in patients with Cushing’s disease.

Individual results (Fig. 3)

Since overall results suggested that the determination of ACTH-RIA and β-endorphin concentrations, and that the calculation of ACTH-RIA/ACTH-IRMA and β-endorphin/ACTH-IRMA molar ratio, could be useful for the distinction between Cushing’s disease and the ectopic...
ACTH syndrome, we compared the individual values of these parameters between the two groups of patients. The upper limit for the ACTH-RIA concentration that was consistent with the diagnosis of Cushing’s disease was 27.8 pmol/l. Three of 17 patients with Cushing’s disease had ACTH concentrations above this threshold, and 4 of 9 patients with the ectopic ACTH syndrome had concentrations below it (Fig. 3A). Thus, the overlap in ACTH-RIA concentrations between Cushing’s disease and the ectopic ACTH syndrome patients was 27%.

The threshold values for the ACTH-RIA/ACTH-IRMA ratio, \( \beta \)-endorphin concentration and \( \beta \)-endorphin/ACTH-IRMA ratio were 2.6, 49.3 pmol/l and 4.5 respectively. The overlap in the ACTH-RIA/ACTH-IRMA ratio (Fig. 3B) and the \( \beta \)-endorphin/ACTH-IRMA ratio (Fig. 3D) were 31% and 27% respectively. The overlap in \( \beta \)-endorphin concentration which was the lowest averaged 19% (Fig. 3C).

No peculiar clinical, biological or etiological common feature could be identified among patients with Cushing’s disease or ectopic ACTH syndrome who overlapped in these parameters except for \( \beta \)-endorphin since only the two patients with Cushing’s disease who exhibited elevated \( \beta \)-endorphin levels showed an obvious pituitary macroadenoma on CT scanning.

Discussion

Plasma ACTH measurement has become indispensable for the diagnosis of pituitary-adrenal disorders. Although several groups developed sensitive RIA (21, 22), most of the commercially available RIA for ACTH measurement in unextracted plasma have a poor sensitivity. This does not permit reliable discrimination between ACTH-dependent and independent causes of Cushing’s syndrome since ACTH can be partially suppressed in Cushing’s disease (e.g. < 4–5 pmol/l) (14, 23). The recent introduction of two-site IRMA has overcome this problem since ACTH concentrations below 1 pmol/l can be measured (3, 4). This is illustrated in our study as plasma ACTH at 08.00 was undetectable in 56% of controls using the RIA while ACTH was measurable in all using the IRMA. ACTH at 08.00 was measurable in only 8% of control subjects with the RIA and in 75% of them with the IRMA. This, together with the high specificity, rapidity and excellent reproducibility of the ACTH-IRMA has led to the recommendation of its preferential use for the investigation of pituitary-adrenal disorders (3, 4).

The specificity of the IRMA may be its only limitation since only ACTH 1–39 forms a “sandwich” complex with the N- and the C-terminal antibodies of the assay. Larger molecular weight precursors or ACTH fragments are not measured and artificially decrease the ACTH-IRMA concentration (4, 24). These variants are preferentially secreted by ectopic tumours (6–11) and their detection in the plasma may aid in differentiating Cushing’s disease from the ectopic ACTH syndrome (5, 12). An indirect but easy way to detect these could be the concomitant use of an ACTH-IRMA with a less specific assay, such as a RIA, which does measure some of these ACTH variants (4). As expected, the ACTH-IRMA values in the ectopic ACTH syndrome and Cushing’s disease patients of our study were similar but the ACTH-RIA concentration was significantly higher in the ectopic ACTH syndrome and was no more correlated...
to ACTH-IRMA values (3–5). Thus, the ACTH-RIA/ACTH-IRMA ratio in the ectopic ACTH syndrome was significantly higher than in Cushing’s disease patients.

However, only 44% of patients with ectopic ACTH syndrome and 17% of Cushing’s disease patients exhibited a clear discrepancy between ACTH-RIA and IRMA concentrations. This may reflect the unspecificity of the ACTH variants which have also been found in some cases of Cushing’s disease (24, 26, 27). Elsewhere, the ACTH antisera used for the RIA, and which has been raised against ACTH 1–24, may not recognize some circulating ACTH variants like corticotropin-like intermediary lobe peptide which arises from the ACTH COOH-terminal end (8). This considerable overlap suggests that, on a single basal plasma sample, the concomitant use of these two ACTH assays is of little help for the etiological diagnosis of ACTH-dependent Cushing’s syndrome.

The processing of proopiomelanocortin gives rise to a number of ACTH-related peptides which can also be used as markers of the secretory activity of the tissue that produce them (28, 29). As ectopic ACTH-secreting tumours more frequently have an altered mode of proopiomelanocortin processing than pituitary tumours, the measurement of these peptides might help in differentiating between Cushing’s disease and the ectopic ACTH syndrome (8–11, 17, 30–32). Only few data concerning plasma β-endorphin concentration

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**Fig. 3.** Comparison of individual plasma hormonal concentrations found in patients with Cushing’s disease (CD) and in patients with the ectopic ACTH syndrome (EAS). The horizontal line drawn within the results of patients with CD represents the mean ± 1 SD of the results obtained in this group. A. ACTH concentration measured using the radioimmunoassay. B. Ratio of ACTH measured using the radioimmunoassay (ACTH-RIA) to ACTH measured using the immunoradiometric assay (ACTH-IRMA). C. β-endorphin concentration. The values mentioned within the graph represent individual β-endorphin results that are outside the range of the graph. D. Ratio of β-endorphin to ACTH concentration measured using the immunoradiometric assay. The value mentioned within the graph represents the individual β-endorphin/ACTH-IRMA ratio result that is outside the range of the graph.
during Cushing’s syndrome are available (15, 18, 33). We used a recently introduced commercial IRMA for \( \beta \)-endorphin which shows an improved specificity and sensitivity compared with most of the previously described assays (18, 34, 35). As expected, Cushing’s disease patients exhibited higher \( \beta \)-endorphin concentrations than controls (36), although the difference did not reach statistical significance. \( \beta \)-endorphin levels in Cushing’s disease were correlated with ACTH levels so that the \( \beta \)-endorphin/ACTH-RIA and -IRMA molar ratios were not significantly different from controls. \( \beta \)-endorphin levels in ectopic ACTH syndrome patients were significantly higher than in Cushing’s disease and did not correlate with ACTH-RIA and IRMA levels. Thus, the \( \beta \)-endorphin/ACTH-RIA and -IRMA ratios increased and were significantly higher than in Cushing’s disease. These observations are basically in accordance with the results of previous studies, carried out for the most part on tumour extracts (10, 15, 17, 18, 35, 37). The dissociation between \( \beta \)-endorphin and ACTH levels in the ectopic ACTH syndrome might result from an alternate processing of the proopiomelanocortin COOH terminal end (16). Alternatively, it might result from differences in metabolic clearance rates of hormones. With regard to this hypothesis, one must note that our \( \beta \)-endorphin assay cross-reacted by 18% with \( \beta \)-LPH (18, 34) which is known to be frankly elevated in the ectopic ACTH syndrome (13, 14, 32) and to have a slower plasma disappearance rate than ACTH and \( \beta \)-endorphin (13, 38).

From a practical point of view, the \( \beta \)-endorphin concentration appeared more useful for discrimination than the other parameters that we studied (overlap = 19%). As the two patients with Cushing’s disease in whom elevated \( \beta \)-endorphin levels were found had an evident pituitary macroadenoma, high levels of \( \beta \)-endorphin favour the hypothesis of the ectopic ACTH syndrome so long as pituitary CT scanning examination is normal. In comparison, the overlap between ectopic ACTH syndrome and Cushing’s disease patients in the cortisol responses to the CRH and dexamethasone tests were only 8 and 12% (data not shown). This emphasizes the well-known superiority of dynamic endocrine testing over a single hormone measurement in this circumstance (39).

In conclusion, our study confirms that ACTH-IRMA levels may be artificially low in the ectopic ACTH syndrome, and that discrepancies between the results of the RIA and IRMA favour the hypothesis of the ectopic ACTH syndrome. However, because of the considerable overlap that exists between Cushing’s disease and the ectopic ACTH syndrome, we do not recommend this comparison in clinical practice. \( \beta \)-endorphin measurement had a better discriminative power, even if it was worse than the results of dynamic testing. Further work concerning the diagnostic power of \( \beta \)-endorphin during dynamic testing is needed to determine the place of its measurement in the hormonal investigation of patients with Cushing’s syndrome.

References

34. Allegro β-endorphin package insert. Nichols Institute. San Juan Capistrano. CA. USA

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