CLINICAL STUDY

A new generation IRMA for ACTH with improved specificity: validation in various physiological and pathological conditions

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Abstract

Objective: Measurement of plasma ACTH is a key step for the exploration of hypothalamic–pituitary–adrenal disorders. To further improve ACTH recognition a new generation of ACTH IRMA was developed using antibodies directed towards succinylated ACTH (sACTH IRMA).

Design: The usefulness of this assay was compared with that of another commercially available ACTH IRMA assay using intact ACTH (ELSA-ACTH) in various pathophysiological situations: patients with low ACTH plasma levels, high ACTH plasma levels with normal or tumoural pituitaries, or ectopic ACTH syndrome, and pregnant women with high proopiomelanocortin (POMC) plasma levels.

Methods: All plasma samples were assayed simultaneously with the two different IRMAs. Comparisons were assessed by plotting the results along the theoretical line of identical values, and by the graphical method of Bland and Altman.

Results: In the ELSA-ACTH IRMA, CLIP (or ACTH18–39) showed true cross-reactivity, and α-melanocyte-stimulating hormone and purified POMC both interfered and induced falsely lower ACTH results; in the sACTH IRMA no peptide showed any cross-reactivity, and only extremely high values of CLIP (50 000 pg/ml) interfered and induced falsely lower ACTH results. In ACTH hypersecretory syndromes, of tumoural (Cushing’s disease, ectopic ACTH secretion) or non-tumoural (Addison’s disease, congenital adrenal hyperplasia) origins a good agreement between the two assays was observed except for very high ACTH plasma values (above 1000 pg/ml) and in some tumours where the sACTH IRMA yielded lower results; in some cases, the presence of circulating CLIP, demonstrated by HPLC studies, may contribute to this discrepancy. It is also likely that the calibration of the ELSA-ACTH kit itself generates higher ACTH values. In normal pregnant women both IRMAs gave highly correlated values, yet lower results were obtained with the sACTH IRMA.

Conclusion: These data show that the sACTH IRMA has improved qualities of specificity and usefulness for rapid assessment of ACTH plasma levels.

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Introduction

Measuring plasma adrenocorticotropicin (ACTH) is a key step for the exploration of hypothalamic–pituitary–adrenal (HPA) disorders (1).

Since the introduction of immunoassays (2), this measurement has become much more sensitive, specific and convenient than with previous bioassays. After the era of RIAs, significant progress was brought about by the IRMA approach, which overcomes most limitations of earlier methods; it obviated the need for plasma extraction, and added a further increase in specificity. Today, the quantification of plasma ACTH is mostly performed routinely by IRMA (3, 4).

In order to further improve the ACTH IRMA an original approach using antibodies directed towards a chemically modified molecule, i.e. succinylated ACTH (sACTH) was recently developed by the Immunotech company (5–6). This strategy yielded two main advantages: first, the generation of monoclonal antibodies with high specificity and affinity, and secondly the stabilization of sACTH in blood and in standard buffer facilitating sample handling and storage. The sACTH IRMA can detect as low as 0.7 pg/ml ACTH in an easy-to-perform two-step assay, with a total incubation time of only 3 h.

We have now explored the usefulness of this IRMA in different states of HPA axis disorders or physiological variations such as pregnancy that may be accompanied by extreme values of circulating ACTH, and/or by the simultaneous presence in blood of intact proopiomelanocortin (POMC), the polypeptide precursor to
ACTH, and/or abnormal fragments such as α-melanocytic-stimulating hormone (MSH) and/or corticotrophin-like intermediary lobe peptide (CLIP or ACTH1-39) (7–15). Indeed, although ACTH IRMAs are highly specific, in that non-ACTH peptides are not measured, they may be flawed by non-ACTH peptides interfering with the assay: high circulating levels of CLIP might compete with and possibly impede the full recognition of ACTH by antibodies that are directed against the C-terminal part of the molecule, and/or α-MSH might titrate the antibodies directed towards the N-terminal part of ACTH. Although, theoretically, none of these peptides (α-MSH, CLIP or POMC…) should be measured by specific IRMAs, all could artificially lower or increase the measured amount of circulating ACTH.

We show here that the sACTH IRMA has unique properties of sensitivity and specificity in all types of pathological or physiological situations (such as pregnancy) which are associated with quantitative or qualitative variations in ACTH or other POMC-peptide secretions. In contrast to some other commercially available IRMAs neither CLIP nor POMC significantly interfered in this new immunoassay for ACTH.

### Patients and methods

#### Patients

Patients were selected on the basis of their particular physiological or pathological conditions, and of their ACTH plasma levels already measured with a commercial ACTH IRMA (ELSA-ACTH) from Cis-Bio International (Gif sur Yvette, France).

**Patients with low ACTH plasma levels** These were 16 patients with undetectable ACTH plasma levels (<2 pg/ml, ELSA-ACTH) and 14 patients with ACTH plasma levels between 2 and 9 pg/ml. These were patients with Cushing’s disease that had been cured by partial hypophysectomy (n = 16), or patients with Cushing’s syndrome of adrenocortical origin (n = 6), one patient with hypopituitarism, and normal responders to a low-dose dexamethasone suppression test (n = 7).

**Patients with high ACTH plasma levels and a normal (non-tumoural) pituitary** These were 13 patients with ACTH plasma levels between 82 and 2034 pg/ml. There were seven patients with Addison’s disease, four with congenital adrenal hyperplasia and two with adrenal cortical cancers treated with oetho, para prime dichloro-diphenyl-dichloroethane.

**Patients with high ACTH plasma levels and pituitary corticotroph tumours (micro- or macroadenomas)** These patients’ ACTH plasma levels varied between 47 and 10 260 pg/ml. There were 14 patients with Cushing’s disease and microadenomas, four with Nelson’s syndrome and three with Cushing’s disease and macroadenomas.

**Patients with high ACTH plasma levels and the ectopic ACTH syndrome** These patients’ ACTH plasma levels varied between 28 and 3150 pg/ml. We tested ten samples from seven patients. There were four bronchial carcinoid tumours and three small cell lung carcinomas.

<table>
<thead>
<tr>
<th>Related peptide</th>
<th>Concentration of added peptide</th>
<th>Concentration of ACTH expected (pg/ml)</th>
<th>Concentration of ACTH measured (pg/ml)</th>
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<td>sACTH IRMA</td>
<td>ELSA-ACTH</td>
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<td>CLIP</td>
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Normal pregnant women  Plasma was obtained from 15 normal women during the third trimester of their pregnancies. All these subjects had normal ACTH plasma levels (17–39 pg/ml). All had highly elevated POMC plasma levels (400–2950 U/ml, normal <100).

Methods
The sACTH IRMA The sACTH IRMA kit was used according to the manufacturer’s instructions (Immunotech, Marseille, France).

Briefly, for assay in duplicate, 500 µl thawed plasma samples, 250 µl alkaline solution and 50 µl succinyllating reagent were dispensed successively into plastic tubes. Tubes were then vortex mixed and incubated for 5 min at 18–25°C.

Three hundred microlitres of succinylated calibrator or succinylated sample were added to coated tubes with two monoclonal antibodies and incubated for 1 h at 18–25°C with constant shaking. The contents of the tubes were then aspirated and 100 µl (3 × 10^5 c.p.m.) per tube of a third radiolabelled monoclonal antibody were added (specific activity at 1.2 mol 125I per mol antibody). After 2 h of additional incubation under the same conditions as above, tubes were washed twice with 2 ml wash solution. The bound radioactivity was then measured.

For the specificity or interference studies, increasing concentrations of synthetic CLIP or purified human POMC (15) were added to plasma samples previously depleted of or containing a known concentration of ACTH. These supplemented samples were then assayed according to the assay procedure.

Dilution studies were performed with samples of all origins under a vast range of concentrations using the zero standard; plasma was diluted 1/3 for the lowest concentrations (17 pregnant women) and 1/10 for higher concentrations.

The ELSA-ACTH IRMA The ELSA-ACTH IRMA kit was used according to the manufacturer’s instructions (Cis-Bio International). It was compared with the sACTH IRMA both for direct plasma assays and for assays in collected HPLC fractions.

HPLC studies Peptides were extracted from plasma (3 ml) on a C18 SEP-PAK cartridge (Waters Associates, St Quentin, En Yvelines, France) with 80% acetonitrile.
in water with 0.1% heptafluorobutyric acid. Aliquots of the collected fractions were lyophilized and reconstituted for HPLC. A 4.6 × 250 mm C18 LiChrospher WP 300 column (Merck, Nogent sur Marne, France) was used with an LKB system (Villepinte, Roissy, France). Absorbance was monitored at 226 nm. Solvent A was milli-Q-water–0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile–0.1% TFA. Using a flow rate of 1.5 ml/min, elution was obtained as follows: starting with a linear gradient from 25 to 35% solvent B up to 35 min, then developing a linear gradient from 35 to 100% solvent B up to 60 min.

Fractions were collected every minute. For IRMA determination, fractions were lyophilized then reconstituted in ACTH-free plasma. The HPLC system was standardized using Peninsula human CLIP and ACTH peptides (Conches, France).

**Statistics**

To evaluate the agreement of the methods of measurement, we used the graphical method proposed by Bland and Altman (16), which focuses on the mean and variability of differences between pairs of measurements. Note that we did not use the correlation coefficient, which is a measure of covariation of the results (how much the values tend to be high or low together) and not a measure of agreement (how much the values tend to be identical). A scatter plot of the difference between the measurements (y-axis) against their mean (x-axis) also allows detection of important lack of individual agreement which may be hidden by the use of global statistics. The plot finally allows investigation of any possible relationship between the measurement error and its true value (estimated by the mean). Plots were made and means and standard deviations of the differences were calculated separately for measurements of plasma ACTH with the two IRMAs.

**Results**

The specificity of the sACTH IRMA has already been described (6). We show here that CLIP, α-MSH, and purified human POMC (hPOMC) are not recognized at very high plasma concentrations (Table 1).

As could be anticipated, some interference of CLIP was observed in the sACTH IRMA, which contributes to diminishing the measured ACTH plasma levels. This interference was only noticed at extremely high concentration levels (50 000 pg/ml): no interference was noticed with α-MSH at a lower concentration of 1600 pg/ml (Table 1). POMC, up to 2000 U/ml did not show any interference effect.

In the ELSA-ACTH IRMA, CLIP showed a significant cross-reactivity, and thus induced an overestimation of ACTH plasma levels. Also, α-MSH interfered in the
assay at the concentration of 1600 pg/ml and lowered measured ACTH concentrations. Purified POMC showed interference which tended to diminish the measured ACTH levels.

For patients with low ACTH plasma levels, identical results were obtained with the two IRMAs; among the 16 patients with undetectable ACTH in the ELSA-ACTH, 15 were also undetectable in the sACTH IRMA (<2 pg/ml).

As shown in Fig. 1, identical ACTH plasma levels were found by both IRMAs in patients with normal (non-tumoural) pituitaries and corticotroph activities elevated for different reasons. In all these patients it is anticipated that intact ACTH is the highly predominant, if not the sole, reactive peptide secreted by the overactive, but otherwise normal, pituitary corticotroph cells. A more precise view of the agreement of the two assays is given by the Bland and Altman representation (Fig. 2). This plot reveals an almost perfect agreement for ACTH concentrations under 1000 pg/ml. For higher values the sACTH IRMA seems to give lower values than the ELSA-ACTH IRMA.

Figure 3 shows the correlation between the two IRMAs in patients with various tumoural reasons for ACTH oversecretion. In general ACTH values were identical with both assays, whatever the type of tumour. The curve shows a tendency for lower results with the sACTH IRMA for very high ACTH concentrations (above 1000 pg/ml). This is again best seen on the Bland and Altman representation (Fig. 4a for mean ACTH under 1000 pg/ml and Fig. 4b for mean ACTH over 1000 pg/ml). In this latter graph (Fig. 4b) note the log scale for the y-axis.

A striking discordant value was obtained in a patient with a large pituitary macroadenoma having ACTH concentrations of 200 and 30 pg/ml with the ELSA-ACTH and the sACTH IRMA respectively (Fig. 3, circled sample). This plasma was submitted to extraction and HPLC, and the fractions were reconstituted and measured with the two IRMAs. Two peaks corresponding to CLIP and phosphorylated CLIP were present and recognized by the sole ELSA-ACTH IRMA, thus explaining that this assay may overestimate the real amount of circulating ACTH; also the ACTH peak appeared better recognized by the ELSA-ACTH IRMA (Fig. 5).

As shown in Fig. 1, both IRMAs gave values that were highly correlated in normal pregnant women, yet slightly lower ACTH levels were obtained with the sACTH IRMA, as shown in the Bland and Altman representation (Fig. 6).

Finally, dilution studies with plasma samples of all origins were performed as indicated in Methods. As shown in Fig. 7, the results confirmed the remarkable reproducibility of the sACTH IRMA.
Discussion

A novel assay has been developed which uses three monoclonal antibodies obtained in mice against a chemically modified human ACTH molecule (5). As already published the sACTH IRMA is rapid (3 h) and offers new improvements in sensitivity and specificity (6). Yet this type of sandwich assay is highly sophisticated and it is important to examine carefully all possible interferences in the assay.
Figure 5 HPLC fractionation of a plasma with discordant ACTH plasma values by the two IRMAs (circled sample of Fig. 3). Each fraction was analysed by the ELSA-ACTH (●) and the sACTH (○) IRMA. The elution volumes of CLIP and ACTH are indicated. Both are preceded by their phosphorylated (p.) counterparts.

Figure 6 Bland and Altman graphical presentation (see Fig. 2) of ACTH assays in pregnant women. Plasma ACTH difference (mean ± s.d.) 8.2 ± 6.4 pg/ml.
Indeed, genuine ACTH is but one among many peptides which result from the complex processing mechanism of its polypeptide precursor, POMC (7). Although ACTH is flanked by two pairs of basic amino acids (Lys-Arg, Lys-Arg), which are two sites of action of the prohormone convertase (PC1), which is specifically present in the anterior pituitary corticotroph cell, there are many situations which alter the complete processing of POMC into intact ACTH. Large amounts of circulating POMC may be present in situations where PC1 function is diminished. That happens in aggressive neuroendocrine tumours which have lost some of their normal neuroendocrine phenotype such as pituitary cancers or macroadenomas of Cushing’s disease, and in small cell carcinoma of the lung responsible for the ectopic ACTH syndrome (15). That was also recently demonstrated in two peculiar conditions: normal pregnant women have very high POMC plasma levels from placental origin (17), and a patient was recently described who had mutations of both PC1 gene alleles with a loss of function inducing very high POMC plasma levels (18).

Because POMC contains the entire ACTH sequence it might be succinylated in the plasma samples and react with the coated antibodies as well. To examine this possible interference we studied the effect of purified hPOMC; at the concentrations used we found no interference indicating that either they were too low or that succinylated POMC did not actually bind to the coated antibodies. It is possible that the ACTH epitopes are hindered within the precursor.

An easy way to check this lack of interference was to observe normal sACTH IRMA values in a population of pregnant women with POMC plasma levels up to 3420 U/ml.

In general, a good correlation was found between plasma ACTH values measured by the new sACTH IRMA and the ELSA-ACTH IRMA (Fig. 1). Yet there was a tendency for slightly lower values obtained with the sACTH IRMA. It is most likely that the calibration of the ELSA-ACTH kit itself generates higher ACTH values as indicated by the slight differences found with the two IRMAs at the ACTH peak after HPLC. We cannot eliminate that a subtle molecular alteration of ACTH, which would not induce a change in retention time, might slightly modify its immunoreactivity preferentially towards one of the two IRMAs.

Other peptides that are released by non-pituitary tumours might also interfere with ACTH IRMAs (13, 14). CLIP contains one of the four succinylated lysines

**Figure 7** Bland and Altman graphical presentation (see Fig. 2) of dilutions in the sACTH IRMA. Plasma ACTH difference (mean ± s.d.) −42.5 ± 136 pg/ml. Note the log scale for mean plasma ACTH (x-axis).
(Lys21). Thus succinylated CLIP might be recognized by one of the coated antibodies, directed against sACTH18-24; it was thus possible that high plasma concentrations of CLIP interfere in the sACTH IRMA and induce artefactually lower values. This effect was indeed observed but only at extremely high CLIP concentrations (50 000 pg/ml). CLIP itself did not cross-react in the sACTH IRMA since the labelled antibody is directed against the extreme N-terminal end of ACTH, and thus cannot recognize CLIP. This is in contrast to what is observed with the ELSA-ACTH IRMA. In this IRMA, the two coated monoclonal antibodies are directed against the N-terminal and central portions of the ACTH molecule. It is thus quite likely that CLIP is recognized by the latter. Indeed, as shown in Table 1, a true cross-reactivity of CLIP was observed. This was confirmed by HPLC study of plasma from a patient with a pituitary macroadenoma; the sACTH IRMA only recognized the ACTH peak, while the ELSA-ACTH IRMA recognized an extra peak corresponding to CLIP. Thus the sACTH IRMA has a definite quality for specificity and, in contrast to some other assays, it should not overestimate ACTH plasma levels in patients who co-secrete CLIP. This latter peptide has no biological action.

As expected, α-MSH had no interference in the sACTH IRMA; indeed, it is a two-step assay where the coated antibodies are directed against the mid- and C-terminal portions of the ACTH molecule, and α-MSH, which cross-reacts with the labelled antibody, was eliminated after the first incubation step. In contrast, α-MSH at 1600 pg/ml induced a slight underestimation of ACTH in the ELSA-ACTH IRMA. It is likely that this peptide interfered with the extreme N-terminal coated antibody of the ELSA-ACTH.

Another maturation product of POMC might, theoretically at least, interfere in the sACTH IRMA: pro-ACTH is the N-terminal fragment of POMC resulting from the action of PC1 at the C-terminal part of ACTH. This peptide thus contains intact ACTH at its C-terminal part. There is evidence that this peptide may be present in blood in some patients with poorly differentiated and aggressive neuroendocrine tumours (19). In this situation, ACTH epitopes might be highly accessible for the coated antibodies. Although the peptide might not be recognized by the third, iodinated antibody, which requires free N-terminal ACTH, it might nevertheless titre the assay capacity and artificially lower the real ACTH plasma levels. In the ELSA-ACTH IRMA this peptide might be recognized by the labelled antibody and also artificially lower the ACTH plasma levels.

We find that the new sACTH IRMA has improved qualities of specificity and usefulness for rapid assessment of ACTH plasma level. Its specificity should augment its capability to better assess the real biological threat of various tumours which may release other non-biochemically active peptides such as CLIP or POMC.

References