A SCREENING TEST FOR LONG-ACTING CORTICOTROPHIN PREPARATIONS

By

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ABSTRACT

The mouse assay for corticotrophin, using plasma steroid levels as the response, is shown to be suitable with slight modifications as a screening test for long-acting corticotrophin. Four hours after the subcutaneous administration of 10–20 milliunits of corticotrophin in saline, the plasma steroid levels had virtually returned to the preinjection levels, whereas corticotrophin injections in combination with 15 per cent gelatine or 1 per cent polyphloretin phosphate, resulted in sustained high plasma steroid levels. Unlike the usual type of prolongation (e.g. insulin, testosterone) which is characterized by delayed maximal end-point readings, peak responses were observed at the same time following prolonged or non-prolonged corticotrophin. Significantly higher responses were observed following long-acting preparations at this time (one hour after the injection). The test also enables the investigator to distinguish between a local and systemic effect of the prolonging material. Thus it was shown that polyphloretin phosphate and gelatine were active only when given in local combination with corticotrophin. The advantages of the method together with its limitations for the assessment of a long-acting effect in man are briefly discussed.

It has recently been shown (Rerup & Hedner 1963) that the mouse is a suitable animal for the assay of corticotrophin. This finding led to the question whether a reliable and efficient screening test for long-acting corticotrophin preparations could be developed.

The questions to be answered by the following study were thus: 1) Is it possible to demonstrate a long-acting corticotrophin effect in the mouse? If so is it possible 2) to demonstrate the site of action and 3) to obtain information with regard to the mode of action of the prolonging agents investigated.
MATERIAL AND METHOD

Albino mice* of either sex weighing about 20 g were used throughout. They were pretreated with 0.2 mg of dexamethasone (»9-α-fluoro-16-methyl-prednisolone«) intraperitoneally at 4 p.m. the day before and at 9 a.m. on the day of the experiment (Rerup & Hedner 1963). Following decapitation blood was collected into heparinized centrifuge tubes. 0.25 ml samples of plasma were analyzed for fluorescent steroid concentration according to Silber et al. (1958) either immediately after centrifugation or after storage in the deep freeze. Normally blood from two randomly chosen mice was pooled in order to obtain sufficient plasma, but pooling of blood from up to 5 mice was performed in the essential screening design. Blood sampling was performed in saline injected control animals and hormone treated animals at different times following subcutaneous injection. In the experiments for evaluating the site of action of the prolonging agents, the first injection was given under the skin of the right hind leg and all other injections were given into the neck region. Solutions were prewarmed to 37°C, the injection volume being 0.2 ml. Polyphloretin phosphate** was used in a concentration of 1 per cent and gelatine*** in a concentration of 15 per cent.

RESULTS

1) Prolonged action. The results of a typical experiment is given in Fig. 1. It may be seen from Fig. 1 that a subcutaneous injection of 10 milliunits of corticotrophin per mouse was followed by a large increase in plasma steroid concentration one hour later. When dissolved in saline, corticotrophin evoked a relatively short-lasting response, which was only just measurable after 3 hours. In combination with polyphloretin phosphate or gelatine, however, steroid levels in the blood were still largely elevated 4 hours after corticotrophin administration (about 60 per cent of the highest response measured). These two agents thus gave rise to a prolonged action of corticotrophin.

Fig. 1 is plotted from single measurements of a plasma pool from 5 mice treated in the same way. Thus no standard error could be drawn and nothing can be stated as to a possible difference between the effects of gelatine and polyphloretin phosphate. No statistics are, however, needed to demonstrate prolongation at the four hour end-point, since the effect of corticotrophin in saline has already disappeared completely at this time. The efficiency of the design is demonstrated by the fact that the necessary information could be obtained from 85 mice involving 17 analyses only. For a screening procedure of a long-acting corticotrophin effect the four hour response is thus considered

* Laboratory Animal Breeding, Laven, Denmark.
** Supplied by Dr. B. Högborg, LEO Ltd., Helsingborg.
*** Prepared from acid treated precursor gelatine, type A, supplied by Charles B. Knox, Cambden, N. J., U. S. A.
Plasma corticosterone level (ordinate) related to time (abscissa) following subcutaneous injections into dexamethasone blocked mice of 10 milliunits of U.S.P. standard corticotrophin in saline (x), 1 per cent polyphloretin phosphate (+), and 15 per cent gelatine (·). Absolute controls are indicated by o.

Fig. 1.

to be optimal because of the greatest difference shown between the prolonged and non-prolonged preparations.

2) Site of action. The site of action of polyphloretin phosphate and gelatine was studied in two separate experiments, the results of which are given in Table 1.

Subcutaneous pretreatment with polyphloretin phosphate or gelatine 30 minutes before the injection of corticotrophin in saline at a different site did not result in an elevated response 4 hours later, which shows that these prolonging agents are active only in local combination with corticotrophin.

3) Mode of action. The time-response curves shown in Fig. 1 are not typical of the prolongation known for other hormones (e.g. insulin, testosterone), where peak responses are observed much later following injection of the long-acting preparation, than after injection of the hormone in saline. The long-acting corticotrophin preparations investigated were, however, at least as active as corticotrophin in saline at the time of the peak activity of the latter, which has been found previously (Rerup & Hedner 1963) to be measurable at about one hour following subcutaneous injection. In addition there was a tendency for long-acting preparations to give a higher response at this
Table 1.
Assessment of the site of action of 1 per cent polyphloretic phosphate (PPP) and 15 per cent gelatine (U.S.P.) on prolonged corticotrophin effect in dexamethasone pretreated mice. Response: Plasma corticosteroid level, µg per 100 ml. Time of action of corticotrophin: 4 hours. Subcutaneous injection of 0.2 ml.

<table>
<thead>
<tr>
<th>Injection into hind leg at 10 a.m.</th>
<th>Injection into neck region at 10.30 a.m.</th>
<th>Number of animals</th>
<th>Blood sampling for plasma steroid analysis at 2.30 p.m.</th>
<th>Response, mean ± s.e.m.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>Saline</td>
<td>5</td>
<td></td>
<td>0.1 ± 0.7</td>
</tr>
<tr>
<td>Gelatine</td>
<td>Saline</td>
<td>5</td>
<td></td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Gelatine</td>
<td>20 mU ACTH in saline</td>
<td>4</td>
<td></td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>No</td>
<td>20 mU ACTH in gelatine</td>
<td>5</td>
<td></td>
<td>35.4 ± 8.0</td>
</tr>
<tr>
<td>No</td>
<td>Saline</td>
<td>5</td>
<td></td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>PPP</td>
<td>Saline</td>
<td>5</td>
<td></td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>PPP</td>
<td>20 mU ACTH in saline</td>
<td>4</td>
<td></td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>No</td>
<td>20 mU ACTH in PPP</td>
<td>5</td>
<td></td>
<td>36.6 ± 7.4</td>
</tr>
</tbody>
</table>

*) Standard error of mean.

Table 2.
Assessment of potentiating effect of polyphloretin phosphate (PPP, 1 per cent) and gelatine (15 per cent, U.S.P.) on corticotrophin after subcutaneous injection into dexamethasone pretreated mice. Response: Plasma corticosteroid level, µg per 100 ml. Time of action of corticotrophin: 1 hour. Injection volume: 0.2 ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10 mU ACTH in saline</th>
<th>10 mU ACTH in gelatine</th>
<th>10 mU ACTH in PPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response</td>
<td>59.0</td>
<td>76.8</td>
<td>79.5</td>
</tr>
<tr>
<td>57.3</td>
<td>67.1</td>
<td>72.5</td>
<td></td>
</tr>
<tr>
<td>52.5</td>
<td>73.6</td>
<td>84.9</td>
<td></td>
</tr>
<tr>
<td>47.1</td>
<td>73.6</td>
<td>92.5</td>
<td></td>
</tr>
<tr>
<td>59.5</td>
<td>82.2</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td>60.1</td>
<td>69.2</td>
<td>89.3</td>
<td></td>
</tr>
<tr>
<td>42.7</td>
<td>75.7</td>
<td>68.7</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± s. e. m.*

\[
\text{Mean} ± \text{s. e. m.}^{*} = 54.0 ± 2.6 \quad 74.0 ± 1.9 \quad 81.2 ± 3.2
\]

\[ t\text{-test versus saline group, } P \]  
\[ < 0.001 \quad < 0.001 \]

*) Standard error of mean.
time (Fig. 1). Three additional experiments involving pooled observations consistently showed higher responses following corticotrophin in gelatine or polyphloretin phosphate than following corticotrophin in saline one hour after the injection. Statistical evidence of a difference at this time was then obtained from three experiments, one of which is given in Table 2.

In this test the difference between gelatine and polyphloretin phosphate was not statistically significant, a finding which was confirmed in subsequent work. The finding that corticotrophin in combination with gelatine or polyphloretin phosphate evoked a clear additional rise in plasma steroid levels over that of the same dose of the hormone in saline at the time of the peak activity of the latter, thus suggests a mode of action due to an inhibition of local hormone destruction, rather than due to a delayed release or absorption from the site of injection. This may also be called potentiation according to pharmacological terminology (Goth 1961).

**DISCUSSION**

The results of this study are in agreement with previous findings obtained in rats and man (Hamburger 1952; Hedner 1963), which showed a clearly prolonged action of corticotrophin in combination with polyphloretin phosphate or gelatine. The period of prolonged action was shorter in mice and rats than in man, a fact which calls for careful interpretation of animal studies with regard to effects in man. The final assessment of the efficiency of a prolonging agent for corticotrophin thus appears to be dependent on the results of an investigation in man. For a screening procedure involving large series of compounds with expected prolonging action on corticotrophin, however, experiments with laboratory animals seem to be indispensable and it is our opinion that for such a screening the four hour mouse test will be a valuable tool because of its efficiency and economy.

With regard to the mechanism of the prolonging effect, it should be remembered that corticotrophin in saline, given subcutaneously, is inactivated locally to a very high degree, since only 1 to 3 per cent of it reaches the blood stream and the adrenal cortex (Rerup 1958). The principle used for long-acting insulin or steroids of delayed release of small amounts of hormone from the injected «depot» would thus a priori be expected not to be very satisfactory for corticotrophin, because of the abundance of corticotrophin splitting enzymes in the tissues. The known properties of polyphloretin phosphate as an enzyme inhibitor and of gelatine as a protector against enzymatic destruction readily explain the unusual type of the time-response curves in Fig. 1. Both agents act against the normally existing high rate of enzymatic destruction without affecting the rate of release of corticotrophin from the injection site to any great extent. This is in agreement with the observed

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higher response at one hour following the administration of long-acting preparations, as compared with corticotrophin in saline, and a subsequently longer biological half life of the prolonged preparation. Thus it appears to us that the prolonging effect of gelatine and polyphloretin phosphate on corticotrophin is not such an effect in the classical sense, i.e. based mainly on delayed release and absorption, but rather based upon the inhibition of corticotrophin destruction at the site of injection. It is hoped that the present test will contribute to the elaboration of new, more prolonged corticotrophin preparations, in which the principle of delayed release of some enzyme inhibitor in combination with the delayed release of corticotrophin at the injection site, could be used with advantage.

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REFERENCES


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