TECHNIC OF ASSAY OF AN UNKNOWN STEROID FOR POSSIBLE LOCAL ACTIVITY IN THE SKIN OF MAN

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With the rapid advances in the field of steroid chemistry and the syntheses of new products, it becomes necessary to have clinical assay technics for evaluation of these new materials. In a discussion of criteria for bio-assay of hormones, Segaloff (1) has listed the following requirements to be met by the ideal assay.

1. Specific
2. Reproducible from laboratory to laboratory
3. Sensitive
4. Statistically sound
5. Simple
6. Rapid
7. Inexpensive

Except perhaps for specificity (as regards a certain steroid), these requirements may apply equally well to the development of technics for the detection of local activity of the corticosteroids. We believe that such an assay should be done in the skin of man, not animals, and should require only small amounts of these new and very precious compounds. The decision to discard a compound or to do more detailed experiments with this compound or group of compounds, is of extreme practical importance.

To assist in the elaboration of such technics, we have used the following program of progressive steps of development, after we have received the steroid from the research chemist and after the initial performance of animal studies for toxicity. It is important to emphasize that we are concerned with attempts to study these compounds at the local or cellular level. Furthermore, such studies, though primarily for assay, may help us to understand the possible mechanism of the local action of these steroids, for Pincus and Hechter (2) have indicated that because of the great interest in these compounds, the problem of such mechanisms has been taken out of the academic field. The skin of man appears more easily suitable to these clinical assay studies than does the eye of man or the joint cavity. As yet, it is not practical to use the nasal mucosa or tissue cultures. The local activity of cortisone in the eye and the failure of local activity of this steroid in the skin of man is well known, and lends support to the argument for the utilization of easily available human skin. The differences of reaction of the steroid locally in the skin of man as compared to the skin of animals (cytologic

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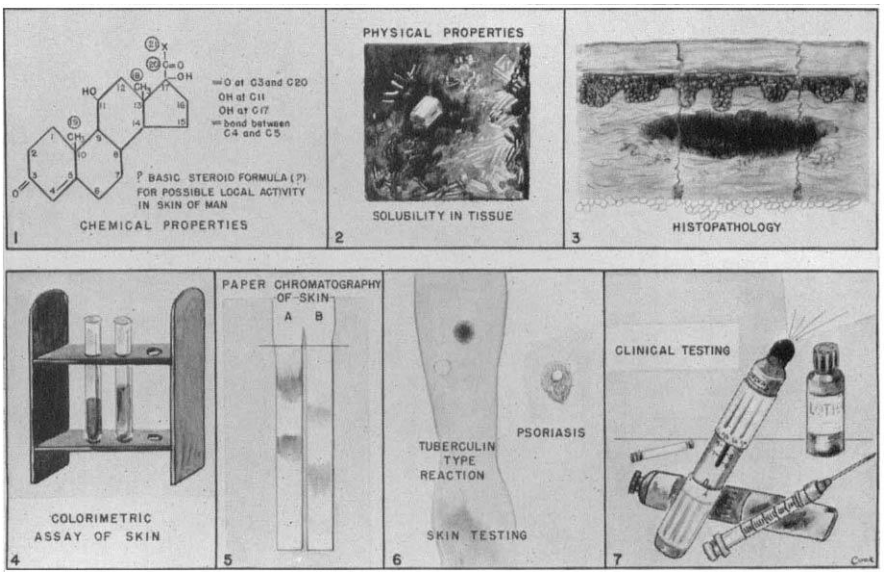


CHART No. 1. Showing the phases of assay technics

changes, granulation tissue, etc.), further emphasizes the need for these studies to be done in man.

In general, the technic of assay includes the following steps in order.

1. estimation of solubility (and or absorbability) in the skin
2. attempts to block, in a more or less standardized fashion, controlled local inflammations
3. controlled clinical experiments with local injections and various types of topical medications

It is the problem for the steroid chemist with his knowledge of the field to determine what formula is to be presented to clinical investigators. Further knowledge on the conversion of steroids in tissue will help him to decide which compounds to select. Most of the compounds now available for clinical assay appear to be derivatives of hydrocortisone.

We must then receive approval from the toxicologist that the materials we are to inject into the tissues of man, in the dosage range selected, are not toxic.

Then, sterile material is suspended in a suitable vehicle. If this is a vehicle which we have not worked with before, then controls must be done with the vehicle. We can proceed with the initial investigative studies with as little as 50 mgs. of an unknown material.

The suspension is injected superficially in the skin of man as an average dose of 2.5 mgs. If this material is relatively insoluble, it must be mixed well and agitated before injection. The local effect of this injection is recorded as regards pain on injection, degree of inflammation, presence or absence of necrosis and duration of the local reaction.

At the end of 24 hours usually, (or sooner, if the material is very soluble in

tissue), a biopsy is taken and one half may be examined in frozen section for crystals and for assay technics, and one half after paraffin embedding and hematoxylin-eosin staining, may be examined for the hematoxylinophilic masses which we have reported (3). Fixation in formaldehyde is preferred to fixation in Helly's to bring out these basophilic masses. Detailed histochemical studies, as to the analysis of these masses, are being continued under the direction of Atkinson (4). Most of the present histochemical studies have been done with hydrocortisone acetate. With this corticosteroid, the mass appears within 24 hours. The mass is made up of desoxyribonucleic acid from the break down of tissue cells bordering the injected site and in the direct area of injection from unidentified mucopolysaccharides, probably of ground substance origin. Hematoxylinophilic masses have been found so far in all of the compounds, in which we have detected some clinical evidence of local activity. Clinically, such areas resulting from the injection of some of less soluble compounds are revealed as atrophic zones which may last for some time depending on the solubility of the injected material. With hydrocortisone acetate, the atrophic portion assumes a poikilodermatous appearance.

With the frozen section technic (5), the location, size and character and persistency of the crystals may be studied. With the red variable Polaroid color filter (Polaroid Corporation), we believe these crystals can be observed more clearly than under ordinary polarized light and can be photographed with greater contrast.

As further studies of the solubility in tissue, we have employed, with additional biopsies, the Porter-Silber colorimetric assay of material from the site of local injection (6). Recently, paper chromatography has been done with skin over which corticosteroids have been rubbed and into which corticosteroids have been injected. This technic also has given additional proof of solubility in tissue by the persistence or disappearance of the material after injection. In order to learn more of the mechanisms at the cellular level, we have also been interested to know if we can detect other steroids which may be formed locally in the skin after injection of a certain steroid. With the small quantities which are available for paper chromatography after the removal of skin biopsies, it has not been possible as yet to show the conversion of the compound injected. These experiments on paper chromatographed are being continued. Our studies of solubility in the skin or absorbability from the skin, are similar to those of Wilson, Glynn, Scull, McEwen and Ziff (7) using the synovial cavity in rheumatoid arthritis. Additional studies are being done in the skin to determine the effect of various enzyme systems on the steroids injected.

With the first step of the assay, we have determined that material is either absorbed rapidly, slowly or not at all. If it is absorbed too rapidly, no local effect may be produced, or the changes may be too rapid to observe (8).

Following these preliminary experiments, we do the studies of "controlled" inflammation in the skin. We have used numerous materials to attempt to produce an uniform tuberculin type of inflammatory response. We have used tuberculin, Frei antigen, mosquito extracts, dust extracts, histoplasmin, tri-

chophyton and oidiomycin. So far, oidiomycin has proved to be the most practical, most "reproducible" and "safe." 0.1 cc of a 1:300–1:500 stock material of oidiomycin is used. At present, we are using oidiomycin (Hollister-Stier Lab.). The patient's response is first determined. The skin reaction for our present dosage of steroid for local inhibition should range only from 5 to 10 millimeters. Reactions which are severe may reveal no recognizable inhibition.

Following the determination of concentration of oidiomycin to be used, the following tests are done, usually on the forearm in order proximally:

(1) oidiomycin + hydrocortisone acetate—2.5 mgs. (as standard)
(2) oidiomycin + unknown steriod (same vehicle and concentration as hydrocortisone acetate)
(3) oidiomycin control

The inhibition at 48 hours is measured and compared with hydrocortisone acetate as the control inhibitor.

Next, we employ the direct intra-lesional injection (usually 2.5 mgs.) into a patch of psoriasis. A disappearance of psoriasis in the area of injection, beginning 2–3 days after injection and definite at the end of 1st week, is a strong presumptuous evidence of local activity in the skin of man. Of all our preliminary clinical testing, this has been the one test easiest to read and most constant in response. With some soluble steroids, the inhibition of inflammation may develop earlier, and may be transient but it does occur.

Only after the experiments listed above, do we go ahead with the study of topical applications. This form of use, Sulzberger (9) has called, aptly, the very practical aspect of local corticosteroid therapy. Since this phase of study demands relatively large amounts of expensive materials, one must have some prior data to warrant such expenditures. Therefore, this portion of the study follows the other phases.

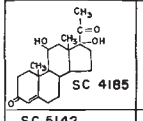
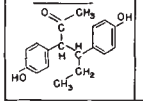
There appears to be two parts to the topical medication assay program.

1. composition of topical medication-concentration; vehicle
2. selection of patients for critical assay studies as opposed to the use of patients in routine clinical surveys

The type of base for the steroid ointment is important. It is possible that the reason for some of our initial inconstant results with hydrocortisone ointment was due to the bases which we employed in the therapy of certain dermatoses. In our experience, a base of mineral oil, white vaseline and lanolin was more constantly effective than a base of the polyethylene glycol type. As Sulzberger (10) has indicated, however, sometimes the reverse is true. Sometimes in certain dermatoses, more lubrication is desired and so the lanolin type is used. It is recommended then that for assay purposes, two types of ointment base be employed. In our experiences, lotions are also effective but these need not be considered in the initial assay program. Moreover, antibiotics and chemotherapeutic agents should not be added to the medications employed in these preliminary studies. Control vehicles should always be used. We believe that the actual clinical investigator and also the patient should not be aware which is the control base and which is the active ointment.

TABLE 1

Showing the detailed summaries of the testing of various compounds, for local activity in the skin of man

COMPOUND	EFFECT OF LOCAL INJECTION				VALUE OF TOPICAL APPLICATION
	HEMATOXYLINOPHILIC MASSES	SOLUBILITY & ABSORBABILITY	INHIBITION OF TUBERCULIN TYPE OF INFLAMMATORY RESPONSE	INHIBITION OF PSORIASIS PATCH	
CORTISONE	+ +	Slow Absorption	0	0	0
CORTISONE TRICARBALLYLATE	+	No Data	0	0	No Data
HYDROCORTISONE FREE ALCOHOL TYPE A	0	Rapidly Absorbed	0	0	No Data
HYDROCORTISONE FREE ALCOHOL TYPE B	+	Absorbed	+	+	+
HYDROCORTISONE ACETATE	+	Slow Absorption	+	+	+
HYDROCORTISONE ALPHA EPIMER	+	No Data	0	0	No Data
 SC 4185	0	No Data	0	0	No Data
SC 5142 (same as SC 4185 with Cl at C9)	+	Slow Absorption	±	Delayed ±	No Data
DESOXYCORTICOSTERONE	0	No Data	0	0	No Data
CHOLESTEROL	0	No Data	0	0	No Data
ESTRADIOL	0	No Data	0	0	No Data
PREGNENOLONE	0	No Data	0	0	No Data
TESTOSTERONE ACETATE	0	No Data	0	0	0
SC 3669 	(Severe Necrosis)	No Data	No Data	No Data	No Data

Care must be exercised in choosing patients for the initial critical assay studies. If the inflammation in the skin is too severe, it may not be able to be blocked. In common, with the clinical testing of other dermatological preparations, efforts should be made to minimize the influence of suggestion. Witten *et al* (11) have indicated, and we have found, the best testing subject is infantile eczema patient, subacute phase without secondary infections. Better control studies are accomplished with the hospitalized infant. Selective areas can be used with control bases on other areas. Another group which can be used, is the group of eczematous dermatitides of the hands and face, chiefly of the eczematous contact type of moderate severity. Rein (12) has also indicated the value of this group. Improvement is produced by the ointment in 48 hours. Following the use of these selected groups for clinical testing, various other conditions may be tried for

routine therapy such as pruritus ani, seborrheic dermatitis and atopic dermatitis, etc. At this stage of the clinical testing, also different concentrations may be tried and also different types of topical medications, such as lotions. At this stage also intra lesional injections into cutaneous lymphoblastomas, localized neurodermatitis, localized scleroderma, sarcoidal lesions, discoid lupus erythematosus may be tried. For local injections into such hard lesions as keloids and Peyronie's Disease (Wayman) (13), we have employed the suspension of the steroid put up in a glass cartridge form or in the metapule for injection with the Hypospray jet injection apparatus.

How have these theoretical considerations been borne out? Table 1 shows some of our findings in the assay of various types of materials. So up to the present, only hydrocortisone, one type of free alcohol and the acetate, have shown the correlation of the positive reports of the preliminary testing with the later positive clinical reports. Today, therefore, just as hydrocortisone is the corticosteroid of preference for systemic administration, so it is the corticosteroid preferred for local use in and on the skin.

These technics are offered then only as a suggestion for preliminary testing before a steroid is recommended for routine clinical use. Future developments will modify and better these technics, for we would agree with Gaddum (14) that "the time has not yet come when the physician is justified in accepting the estimate of hormones without a critical consideration of the methods by which these estimates have been obtained."

CONCLUSIONS

A technic of bio-assay is described in which quantities as little as 50 mgs. of a new unknown steroid can be evaluated as to potential local effect in the skin of man. Only after these preliminary experiments, should the more extensive and expensive detailed clinical studies be initiated, first, with controlled critical studies with injections and topical applications, and then routine clinical surveys. In our experience, only hydrocortisone free alcohol and hydrocortisone acetate have shown the correlation of the preliminary assay technic, and the subsequent clinical value for local activity in the skin of man.

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