responded in a somewhat similar manner as the PEG ointments. Relatively low salicylate blood levels were obtained with this gel, but it appeared that the blood levels obtained were somewhat higher than with PEG ointment. This may be due to a less intense complexation occurring in the case of the surfactant polymer than in the case of PEG.

SUMMARY

1. Dimethyl sulfoxide, in a 15% concentration, enhanced the percutaneous absorption of salicylic acid from hydrophilic ointment USP XVII and hydrophilic petrolatum USP XVII.

2. Dimethyl sulfoxide, in a 15% concentration, hindered the percutaneous absorption of sodium salicylate from hydrophilic ointment USP XVII.

3. Salicylic acid is slowly released from polyethylene glycol ointment USP XVII and a polyoxyethylene (20) stearyl ether gel system.

4. Dimethyl sulfoxide, in a 15% concentration, had little effect upon the release of salicylic acid or sodium salicylate from polyethylene glycol ointment USP XVII.

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Keyphrases

Salicylic acid, sodium salicylate absorption—ointments
Dimethyl sulfoxide effect—salicylic acid, sodium salicylate absorption
Ointment preparation—salicylic acid, sodium salicylate
Colorimetric analysis—spectrophotometer

Analysis of Steroids VIII

Determination of Conjugated Ketosteroids in Pharmaceutical Preparations Using the Sodium Borohydride Method

By SÁNDOR GÖRÖG

A simple and rapid ultraviolet spectrophotometric method has been developed for the determination of Δ^4-3-keto- and $\Delta^{1,4}$-3-ketosteroids in pharmaceutical preparations. The method consists of the reduction of the C-3 carbonyl group with sodium borohydride, followed by the determination of the decrease of absorbance due to the reduction measured by differential spectrophotometry. Other active components or excipients of the preparation whose absorbances are not changed by sodium borohydride do not interfere with the determination.

Δ^4-3-KETO AND $\Delta^{1,4}$-3-KETO bonding systems very often occur in various kinds of steroid drugs. Their simplest determination is carried out spectrophotometrically. In some cases the direct spectrophotometric measurement can be done without any difficulties with the aid of the intensive absorption band at about 241 mμ, log $\epsilon = 4.2$ (1).

The spectrophotometric determination is disturbed either by the spectra of other components present in the pharmaceutical preparation or by the excipients if they have light absorption. In

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such cases the spectrophotometric measurements are generally combined with chromatographic separations (2), or the spectrum is shifted toward the longer wavelengths by means of a reaction by one of the condensation types, where selective measurements can be made (3, 4).

In this paper a new method is presented which permits the quantitation of conjugated ketosteroids in the presence of accompanying substances with absorbing properties. A method for the specific spectrophotometric determination of conjugated ketosteroids (5) has been recently published. The method essentially is the reduction of the C-3 carbonyl group of the Δ^4-3-ketosteroids with sodium borohydride. This results in the disappearance of the conjugation band in the ultraviolet spectra of the compound. Thus, if the spectrum of the test substance is taken and a solution of the same concentration as the sample is used as a blank, but treated with sodium borohydride, the absorbance measured at about 241 mμ gives the contents of conjugated ketosteroids, even in the presence of impurities showing absorption characteristics, provided that the latter do not change their spectra on treatment with sodium borohydride. In this case the light absorption due to the impurities is cancelled out, and the measured absorbance is characteristic of the contents of conjugated ketosteroids.

This principle has been successfully applied to check the contents of active ingredients in a number of pharmaceutical preparations.

EXPERIMENTAL

The assay of some characteristic examples from among the preparations of Chemical Works of G. Richter Ltd., Budapest, will be described.

Reagents and Apparatus—The sodium borohydride and solvents, methanol and ethanol, used were of analytical grade. Sodium borohydride was weighed into the reaction flask as a solid; in each instance 0.1 g. ± 5% was used. Hydrochloric acid, 1 *N*, 0.01 *N*, aqueous solution. Sodium hydroxide, 1 *N*, aqueous solution. Spectromom 202 spectrophotometer was used in this study.

Determination of 19-Nortestosterone Phenylpropionate in Oil-Injectable Formulations—Thoroughly shake 1.00 ml. of the injection solution, containing 25 mg. of the active ingredient,[1] for 5 min. with 100 ml. of methanol and allow to stand until the oil has settled. Transfer 5 ml. of the stock solution to a 100-ml. volumetric flask, add 5 ml. of methanol followed by 0.1 g. of sodium borohydride. After the sodium borohydride has been dissolved allow the mixture to stand for 15 min. and dilute to volume with methanol.

Weigh 0.1 g. of sodium borohydride into another 100-ml. volumetric flask. Allow to stand with 10 ml. of methanol for 15 min. and then boil for 2 min. After cooling add 5 ml. of the stock solution and dilute to volume with methanol. Determine the absorbance of this solution at 240 mμ using the former solution as a blank.

The concentration of the active ingredient in the sample is calculated from the following equation:

$$\text{mg. of 19-nortestosterone phenylpropionate per ml. formulation} = 2000A/a$$

where a = 42.1, the absorptivity of the active ingredient at 240 mμ.

Determination of Progesterone in Injectable Formulations[2]—*Composition A*—An oil solution containing 12.5 mg. of progesterone and 2.5 mg. of estradiol monobenzoate per ml. with benzyl alcohol as the preservative.

Treat 2 ml. of the injection solution exactly as described above for 19-nortestosterone phenylpropionate.

The concentration of progesterone is calculated as follows:

$$\text{mg. of progesterone per ml. formulation} = 1000A/a$$

where a = 54.1, the absorptivity of progesterone at 240 mμ.

Composition B—An aqueous solution containing 12.5 mg. of progesterone and 2.5 mg. of estradiol monobenzoate per ml. and containing emulsifiers.

Dilute 2 ml. of the injection with sufficient methanol to make 100 ml. Treat this stock solution exactly as described above for 19-nortestosterone phenylpropionate. Determine the absorbance at 250 mμ.

The concentration of progesterone is calculated from the equation for Composition A. In this case a = 42.7, the absorptivity of progesterone at 250 mμ.

Determination of Prednisolone in Ointments—The contents are 0.5% prednisolone and 0.2% methyl *p*-hydroxybenzoate in a hydrophilic ointment.

Dissolve 2.5 g. of the formulation by heating with 50 ml. of ethanol. Dilute the solution with ethanol to make 100.0 ml. To a 10-ml. aliquot of this stock solution add 1 ml. of 1 *N* sodium hydroxide and 0.1 g. of sodium borohydride and reflux for 1 hr. After cooling add 5 ml. of 1 *N* hydrochloric acid and dilute the solution with ethanol to make 100 ml.

Carefully add 5 ml. of 1 *N* hydrochloric acid to a mixture of 0.1 g. sodium borohydride and 1 ml. of 1 *N* sodium hydroxide. When the evolution of hydrogen has ceased, add 10 ml. of the stock solution and dilute with ethanol to 100 ml. Determine the absorbance of the solutions at 243 mμ, using the solution boiled with sodium borohydride as the reference.

The prednisolone content is calculated as follows:

$$\% \text{ prednisolone} = 100A/a$$

where a = 41.0, the absorptivity of prednisolone at 243 mμ in ethanol solution and W is the weight in g. of the sample.

[1] Nerobolil, Richter tradename.

[2] Limovan, Richter tradename.

TABLE I—SPECTROPHOTOMETRIC ANALYSIS OF STEROIDS IN DIFFERENT FORMULATIONS

Formulation Investigated	No. of Tests	Contents of Active Ingredient Found[a]	Declared
19-Nortestosterone phenylpropionate in oil injection	6	24.62 ± 0.17 mg./ml.	25 mg./ml.
Progesterone in oil injection	4	12.42 ± 0.10 mg./ml.	12.5 mg./ml.
Progesterone in aqueous emulsion injection	5	12.66 ± 0.24 mg./ml.	12.5 mg./ml.
Prednisolone in ointment	7	0.491 ± 0.0059%	0.5%
Prednisolone in tablets	5	1.975 ± 0.014 mg./tab.	2 mg./tab.

[a] Average ± standard deviation.

Determination of Prednisolone in Tablets—The active ingredients are 100 mg. of phenylbutazone and 2 mg. of prednisolone per 0.3-g. tablet.[3]

Extract 1 g. of the finely pulverized tablet by shaking for 2 hr. with 60 ml. of 0.01 *N* hydrochloric acid. Filter the extract into a 100-ml. volumetric flask, wash with 3 × 10 ml. of 0.01 *N* hydrochloric acid, and dilute to volume with 0.01 *N* hydrochloric acid. To a 10-ml. aliquot of the stock solution add 2 ml. of 1 *N* sodium hydroxide followed by 0.1 g. of sodium borohydride. Heat the solution for 1 hr. on a steam bath. After cooling add 4 ml. of 1 *N* hydrochloric acid and dilute the solution with water to make 50 ml.

Dissolve 0.1 g. of sodium borohydride in 10 ml. of water in another 50-ml. volumetric flask. Carefully add 4 ml. of 1 *N* hydrochloric acid followed by 2 ml. of 1 *N* sodium hydroxide and 10 ml. of the stock solution. Dilute to volume with water. The absorbance of the solution is determined at 248 mμ against the solution heated with sodium borohydride.

The following equation is used for the calculation:

$$\text{mg. of prednisolone per tablet} = \frac{500A \times \text{av. wt./tablet}}{a \times \text{wt. of the sample}}$$

where a = 39.7, the absorptivity of prednisolone at 248 mμ in aqueous solution.

RESULTS

Table I summarizes typical analyses of five formulations.

From the data of Table I it is seen that the standard deviation does not usually exceed ±1% with the exception of the aqueous emulsion containing progesterone, where the standard deviation has increased 1.9% owing to the difficulties described in the *Discussion*, and the difference from the declared contents of active ingredients is less than ±2%.

DISCUSSION

In every case stock solutions were prepared by adequate extraction of the formulations. An aliquot was taken from the stock solution and submitted to reduction by sodium borohydride. This solution was used as a blank. The test solution was prepared by adding an aliquot of the same volume of the stock solution to sodium borohydride previously decomposed either with hydrochloric acid or by boiling with methanol, and the solution obtained was diluted in the same way as the blank.

The reduction of Δ^4-3-ketosteroids was accomplished according to the method described previously (5): in methanol solution, with a large excess of sodium borohydride, at room temperature for 15 min. With $\Delta^{1,4}$-3-ketosteroids only a partial reduction takes place under such conditions. For this reason, in the case of formulations containing prednisolone, the reduction was carried out at the boiling point. Since sodium borohydride decomposes very quickly in methanol under these conditions, ethanol or water was used instead of methanol as a solvent and sodium hydroxide was employed as a stabilizer. In this manner a quantitative reaction can also be achieved in the above-mentioned cases.

The pH value of the test solution does not affect the results. Therefore, in the case of Δ^4-3-ketosteroids the measurements were made in mild alkaline solution produced by the decomposition products of sodium borohydride. In the case of prednisolone in aqueous medium, the measurements were made in the solution which had been made alkaline to stabilize the sodium borohydride; in ethanolic medium, owing to the poor solubility of the decomposition products of sodium borohydride in alkaline ethanol, the measurements were made after acidifying with hydrochloric acid.

The only prerequisite to obtaining correct results in this manner is that the other active ingredients and excipients used should not change their absorbance at about 241 mμ on treatment with sodium borohydride. This condition was fulfilled with the preparations studied.

Special mention must be made of the oil, *Oleum helianthi*, very often used as a solvent for steroid hormones. The active ingredient of the oil injection was extracted simply by shaking the injection with a 50–100-fold quantity of methanol. After the separation of the oil, the methanol solution was tested. It was found that the active ingredient can be completely extracted by a single extraction.

However, other substances showing absorption are also extracted from the oil by methanol. Therefore direct measurement is impossible even in the absence of other absorbing components (19-nortestosterone phenylpropionate injection). Curve a, Fig. 1, indicates the spectrum of this formulation; it was taken after extraction and dilution (as described in *Experimental*) but without treatment with sodium borohydride. It can be seen that the spectrum shows large distortion in comparison with the spectra of Δ^4-3-ketosteroids. Curve b is the spectrum of the solution reduced with sodium borohydride, showing the spectrum of the substances extracted from the oil (background), while the differential spectrum (Curve c) which was taken as described in *Experimental*, is in good agreement with the spectrum of 19-nortestosterone phenylpropionate (6).

There is only a small irregularity in the differential

[3] Rheosolone, Richter tradename.

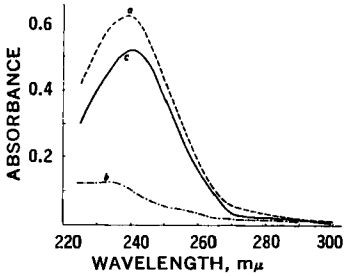

Fig. 1—Determination of 19-nortestosterone phenylpropionate in oily injection. Key: a, spectrum of diluted stock solution without treatment with sodium borohydride; b, spectrum after treatment with sodium borohydride; c, differential spectrum of the solutions a and b.

curve, namely, the shoulder of small intensity that occurs at about 280 mμ, where 19-nortestosterone phenylpropionate has no measurable absorption at this concentration.

The differential spectra of the methanolic extracts of various oil samples treated with sodium borohydride were recorded to clarify this question. It was found that a differential spectrum appears with a small but reproducible intensity. The maximum of this spectrum is at about 280 mμ, and the differential absorbance measured at 240 mμ can be neglected under the given conditions.

This effect can be more distinctly seen on Curve c, Fig. 2, where the differential spectrum of the oil-injectable formulation of progesterone is presented. In this case the concentration of the active ingredient is half that in the case of 19-nortestosterone phenylpropionate injection, and thus the relative concentration of impurities is doubled. In this case the error was not yet significant, but oily injections containing smaller quantities of active ingredient cannot be tested with the desirable precision. The absorption band, $\lambda_{max.}$ 230 mμ (7), of estradiol monobenzoate and the aromatic fine structure of benzyl alcohol $\lambda_{max.}$ 252, 258, 264, 268 mμ, can readily be recognized on Curve b, Fig. 2. The latter also can be seen on Curve a.

In the cases described above the background eliminated by differential spectrophotometry at 240 mμ did not amount to 50% of the differential

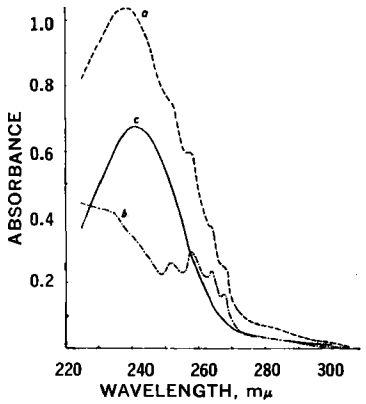

Fig. 2—Determination of progesterone in oil injection. See Fig. 1 for key.

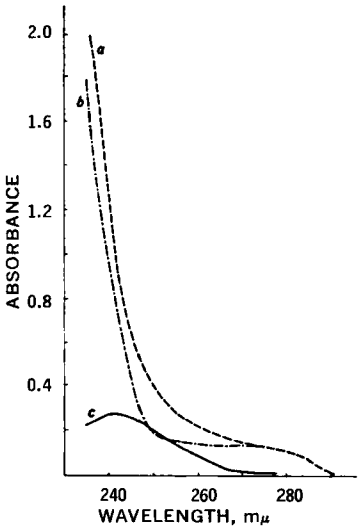

Fig. 3—Determination of progesterone in aqueous emulsion injection. See Fig. 1 for key.

absorbance. However, the measurement could also be accomplished with the aqueous emulsion injection containing progesterone, where the value of the background (Curve b, Fig. 3), owing to the auxiliaries used in the preparation of the emulsions, is a multiple of the value of the differential spectrum (Curve c). In this case the measurement, because of the high absorbance of the blank, was not made at the peak but at the slope of the spectrum of progesterone, namely at 250 mμ. The error in this case slightly exceeded that observed with the other preparations tested.

In the case of the prednisolone-containing ointment, the interfering effect of methyl *p*-hydroxybenzoate had to be prevented. The spectrum of this compound [$\lambda_{max.}$ 258 mμ, a = 109.2 (8)] can be identified in Curve b, Fig. 4. The differential spectrum (Curve c) shows appropriate agreement with the spectrum of prednisolone.

In the test with tablets containing prednisolone and phenylbutazone, the problem was due to the

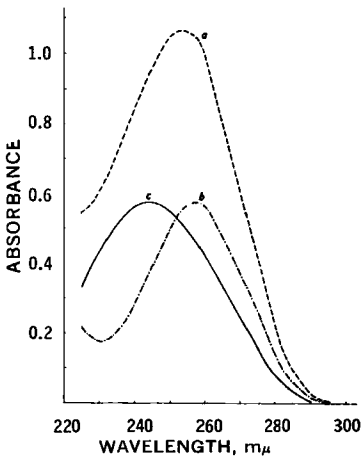

Fig. 4—Determination of prednisolone in ointment. See Fig. 1 for key.

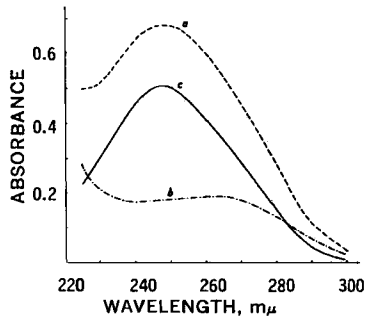

Fig. 5—Determination of prednisolone in tablets. See Fig. 1 for key.

presence of a 50-fold quantity of phenylbutazone having nearly the same absorbance at about 240 mμ as the prednisolone. In the presence of both active ingredients (alcohol extraction), the measurements, owing to the high background absorbances, could only be made in dilute solutions. The sensitivity and precision of the method was extremely decreased by this circumstance. To avoid this, an attempt was made to select the extraction. This could be achieved by the use of a 0.01 *N* solution of hydrochloric acid. In this solution prednisolone dissolves satisfactorily for the purpose given, while phenylbutazone being present at this acidity quantitatively in the keto form is dissolved only slightly. Thus the extraction of prednisolone becomes nearly selective. The small amount of phenylbutazone extracted has carbonyl groups of acid hydrazide type, which do not react with sodium borohydride, therefore the differential curve (Curve c, Fig. 5) is suitable for the correct estimation of prednisolone. The background (Curve b) is characteristic of the spectrum of phenylbutazone measured in alkaline solution [$\lambda_{max.}$ 264 mμ (9)].

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Keyphrases

Steroids—analysis
Ketosteroids, conjugated—determination in pharamaceuticals
Sodium borohydride reduction—steroid analysis
UV spectrophotometry—analysis

Effect of Physostigmine upon the Output of Catecholamines from the Adrenal Gland of the Rat

By C. L. KAUL and R. S. GREWAL

An investigation has been made on the effect of physostigmine on the output of catecholamines from the adrenal gland of the rat. Physostigmine (20 mcg. i.v.) causes three- to fourfold increase in the catecholamines output from the adrenal gland of the rat. This effect is mediated centrally as no increase was seen in the pithed animals. It is concluded that this peripheral release of catecholamines does not play any significant role in the hypertensive response of physostigmine in the rat.

INTRAVENOUS administration of physostigmine causes an appreciable rise in blood pressure in an urethan-anesthetized rat (1, 2). This pressor response of physostigmine has been mainly attributed to central adrenergic stimulation and is absent or much less in pithed animals (2). Although the hypertensive response of physostigmine is mainly central, there is some evidence to suggest that some peripheral action may also be involved (2–4). Medaković and Varagić (5) have also postulated that liberation of epinephrine and norepinephrine from the adrenals does not seem to play a significant role in the hypertensive response to physostigmine, as the response to physostigmine was the same in normal or adrenalectomized animals.

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