THE SPECTROPHOTOMETRIC DETERMINATION OF αβ-UNSATURATED ALDEHYDES AND KETONES WITH GIRARD-T REAGENT

PART II. KETOSTEROIDS

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A rapid spectrophotometric method is suggested for the determination of ethisterone and of methyltestosterone in tablets by reaction with Girard-T reagent.

THE principle of the method described by Stenlake and Williams[1] is now applied to the determination of αβ-unsaturated ketosteroids, in particular, ethisterone and methyltestosterone in tablets. Assays for these tablets are not given in the British Pharmacopoeia and the methods described in the United States Pharmacopeia XV involve long extraction procedures. Hot glacial acetic acid which has been used as a solvent in the preparation of Girard-T hydrazones[2,3] readily extracted the ketosteroids from tablet bases, to give a solution suitable for treatment with Girard-T reagent. As expected[2–4], reaction was complete within three minutes at 100° with both ethisterone and methyltestosterone, and this was adopted as the standard reaction time. The stability of the final solutions and agreement with the Beer-Lambert law was satisfactory.

Preliminary observations showed that sucrose and glucose interfered to a marked extent when present as solids in the reaction mixture. This interference was reduced when solutions which were saturated at room temperature with respect to the sugars were used. Two methods are therefore described, a general method applicable to tablets of unknown composition, and a direct one which can be used when the tablet basis is known, and does not contain glucose or sucrose.

EXPERIMENTAL

Standards

Ethisterone was recrystallised from ethanol. After drying at 105° for 2 hours the crystals had the following constants: m.p. 271–274°, $[\alpha]_D^{17°}$ + 33° (*c*, 1·1 pyridine), *E* (1 per cent, 1 cm.) 766 at 282 mμ when *determined by the method described.* The corresponding *E* (1 per cent, 1 cm.) values for the anhydrous manufacturing samples of ethisterone were: Batch 1: (i) 762, (ii) 762; Batch 2: 759.

Methyltestosterone was recrystallised from aqueous ethanol. After drying at 105° the crystals had the following constants: m.p. 165·5–167°, $[\alpha]_D^{17°}$ + 84·5° (*c*, 1·02 in ethanol), *E* (1 per cent, 1 cm.) 771 at 283 mμ when *determined by the method described.* The anhydrous manufacturing sample gave *E* (1 per cent, 1 cm.) 768 at 283 mμ by the same method.

Method for the Determination of E (1 *per cent*, 1 *cm.*) *Constants*

Dissolve the ketosteroid (about 75 mg. accurately weighed) in glacial acetic acid and adjust to 50 ml. To the solution (1 ml.) in a dry test-tube add Girard-T reagent (20 mg.). Plug the tube loosely with cotton wool and place in a boiling water bath for 3 minutes, swirling the tube gently at first to ensure solution of the reagent. Cool, dilute with water and transfer immediately to a 200 ml. standard flask which contains water (100 ml.) and sufficient N sodium hydroxide to neutralise 9/10 of the acetic acid. Wash out the tube and add the washings to the flask. Adjust to volume with more water, mix well and measure the optical density of the solution in 1 cm. cells at the appropriate wavelength of maximum absorption using water as reference solution. Repeat the operation, omitting the ketosteroid. Calculate the *E* (1 per cent, 1 cm.) constant from the difference between the optical densities.

General Method for Tablets

Take a sample of 20 tablets and determine the average weight. Powder the tablets and transfer an accurately weighed quantity, equivalent to about 10 mg. of ketosteroid, to a dry test-tube. Add about 7 ml. glacial acetic acid, and heat in a boiling water bath for 2 minutes, stirring the mixture thoroughly. Cool the mixture and filter through a small plug of cotton wool directly into a 10 ml. graduated flask using slight suction. Treat the residue with three successive quantities of glacial acetic acid each of 1 ml. as before, cool and pass the extract through the filter. Finally extract the residue with glacial acetic acid, 3 ml., cool and filter into a separate container. Use this filtrate to adjust the contents of the standard flask to volume and reserve the remainder for use in the preparation of a blank solution. Continue as described under Method for Determination of *E* (1 per cent, 1 cm.) Constants from "To the solution (1 ml.) . . ." but using as a blank 1 ml. of the reserved portion of glacial acetic acid treated in the same way. Calculate the concentration of ketosteroid in the tablet using the appropriate constant.

Direct Method for Tablets

Take a sample of 20 tablets and determine the average weight. Powder the tablets and transfer an accurately weighed quantity, equivalent to about 1 mg. of ketosteroid, to a dry test-tube. Add 1 ml. of a freshly prepared 2 per cent w/v solution of Girard-T reagent in glacial acetic acid and proceed as described in the Method for the Determination of *E* (1 per cent, 1 cm.) Constants from "Plug the tube loosely . . .", using as a blank the appropriate quantity of tablet basis treated in the same way. Calculate the concentration of ketosteroid in the tablets using the appropriate constant.

Efficiency of Extraction

A mixture of ethisterone (17·7 mg.), acacia (15 mg.) and lactose (0·4 g.) was treated as described under General Method for Tablets. The recovery of ethisterone was 100 per cent.

The extracted tablet residues from two determinations on Batch P (Table II) were bulked, boiled with glacial acetic acid (4 ml.) for 1 minute and digested at 100° for 5 minutes. The mixture was cooled, filtered and the filtrate (1 ml.) treated as described under Method for Determination of *E* (1 per cent, 1 cm.) Constants. A normal blank value only was obtained.

The extracted tablet residue from one of the assays was dissolved in water and the insoluble portion, after separation, was treated as described under Direct Method for Tablets from "Add 1 ml. . . .". No absorption corresponding to $\alpha\beta$-unsaturated ketosteroid was obtained.

Effect of Tablet Bases

The substances (see Table I for names and quantities) were treated as described under General Method for Tablets. The optical densities obtained using distilled water as reference solution are recorded in Table I.

TABLE I

EFFECT OF TABLET BASES

Substance	Quantity (g.)	Optical density
Reagents	—	0·020
Lactose	0·3	0·025
Mannitol	0·3	0·020
Dextrin	0·3	0·015
Tragacanth	0·05	0·020
Sucrose	0·3	0·070, 0·074
Glucose..	0·3	0·058

Lactose (0·05 g.) was treated as described under the Direct Method for Tablets. The optical density obtained was 0·04 using distilled water as reference solution. Sucrose (0·05 g.) was treated as for lactose above. The optical density found was 0·18.

Examination of Ethisterone Tablets Batch AA

The powdered tablets (4·475 g.) were extracted continuously for 4 hours with light petroleum, the extract evaporated and the residue (A, 8·1 mg.) reserved. Extraction was continued for a further 4 hours with chloroform, the extract was evaporated and the residue (B) dried to constant weight at 105° (yield 0·1225 g.). From residue (B) ethisterone in a tablet of average weight = 5·53 mg. or 110·5 per cent of labelled strength.

Examination of Residues A and B by the Girard-T Spectroscopic Method

Residue A was dissolved in 5 ml. of glacial acetic acid, and 1 ml. treated as described under Determination of *E* (1 per cent, 1 cm.) constants. Ethisterone found: 43·5 per cent (equivalent to 3·5 mg.). Residue B was dissolved in glacial acetic acid and made up to 100 ml. with more glacial acetic acid. The solution (1 ml.) was treated as described under Determination of *E* (1 per cent, 1 cm.) Constants. Ethisterone found: 86·3 per cent (equivalent to 105·8 mg.). Total ethisterone: 109·3 mg. equivalent to 4·93 mg./tablet, or 98·6 per cent of labelled strength.

Results and Discussion

The results obtained by the Girard-T spectroscopic assay of ethisterone and methyltestosterone in tablets are recorded in Table II. The speed with which the method can be applied represents a considerable advance on the U.S.P. XV method for ethisterone, for which the extraction procedures alone require 8 hours.

TABLE II

Girard-T spectroscopic assay of ethisterone and methyltestosterone

Tablets	Batch	Strength (mg.)	Found (mg.)	Label strength per cent	Label* strength per cent
Ethisterone	P	5	4·72	94·4	104
			4·79	95·8	
			4·74	94·8	
,, ...	Q	5	4·83	96·6	96·5
,, ...	R	10	9·1	91·0	91
,, ...	S	10	9·35	93·5	102
,, ...	T	10	9·12	91·2	95·7
			9·14	91·4	
,, ...	U	10	9·58	95·8	100
,, ...	V	10	9·66	96·6	102
,, ...	W	25	23·6	94·4	97
,, ...	X	25	23·7	94·8	101
			23·5	94·0	
,, ...	AA	5	4·91	98·2	100·4
			4·93	98·6	
Methyltestosterone	Y	3·6	3·50	97·2	100
,, ...	Z	3·6	3·54	98·3	97

* Results supplied by Organon Laboratories Ltd.

Although the results (column 4) appear to show low recoveries, control experiments on extracted tablet basis showed no unextracted ketosteroid. Recovery was also quantitative from a mixture of known composition. Two factors contribute to the apparently low figures, one being the fact that our constants were determined on recrystallised anhydrous samples of ethisterone and methyltestosterone. These give slightly higher E (1 per cent, 1 cm.) values than the manufacturing samples examined. The actual manufacturing samples used in the production of the various batches of tablets were not available to us, so that correction of the results was not possible Secondly some differences were found between the average weight of the tablets and the theoretical manufacturing weight, and corrections for these are incorporated in the Organon Laboratories results which are quoted in column 6 by kind permission of Dr R. P. Edkins. These results, obtained by a different method, show reasonable agreement with our own in several instances but notable discrepancies exist.

The results obtained on the batch AA of ethisterone tablets provide an interesting comparison of the Girard-T spectroscopic and the U.S.P. XV extraction methods. The latter, which is based on a gravimetric determination of chloroform-soluble matter after a preliminary extraction with light petroleum, gave a high result equivalent to 110·5 per cent of labelled strength. Application of the Girard-T spectroscopic method to the chloroform-soluble residue showed that only 86·3 per cent of this extract, equivalent to 95·4 per cent of the labelled strength, was ethisterone. It is significant that a similar examination of the light-petroleum-soluble

extractive showed that 43·5 per cent, equivalent to 3·16 per cent of labelled strength, was ethisterone, giving a total yield by this method equivalent to 98·6 per cent of the labelled strength.

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REFERENCES

1. Stenlake and Williams, *J. Pharm. Pharmacol.*, 1957.
2. Wolfe, Hershberg and Fieser, *J. biol. Chem.*, 1940, **136**, 653.
3. Morris and Williams, *ibid.*, 1953, **54**, 470.
4. U.S.P. XV, p. 276.

DISCUSSION

The papers were presented by DR. W. D. WILLIAMS.

The CHAIRMAN said that it was necessary not only to determine how much $\alpha\beta$-unsaturated ketone was in the tablet, but also to identify it. The authors had emphasised that the extinctions were almost identical for the two compounds and, therefore, the active ingredient must be isolated.

MR. H. B. HEATH (Sudbury). Had the authors any comment on the application of the method to determinations of the citral content of solutions in 40 per cent ethanol or *iso*propyl alcohol?

MR. A. R. ROGERS (Brighton). Had the authors checked that extinction was proportional to path length?

MR. S. G. E. STEVENS (London) referring to Table II of Part I asked how a deviation from the quantities quoted would affect accuracy of the answer? Were Stafford Allen's samples tested at the same time, or were they oils which had been stored and had possibly undergone some degree of resinification?

Under "Efficiency of Extraction", in Part II, the authors had used a mixture containing acacia and stated that the recovery was 100 per cent. One would have thought a number of saccharides would probably have been produced, which may have caused the ill effects reported in the case of glucose and lactose, and it would be difficult to get a 100 per cent recovery.

DR. W. MITCHELL (London). The method took considerably longer to perform than the method of the British Pharmacopoeia and the results with lemon oil were consistently much lower. It was desirable to test more samples before drawing any final conclusions. There might be a slight error in the citral standard, because it was difficult to get it completely free from methyl heptenones. The fall in results of so-called pure citral might be explained by the production of methyl heptenone on subsequent storage.

DR. G. E. FOSTER (Dartford). Was it necessary to make a calibration curve with each batch of reagent? Had any work been done on the injections of the hormones?

The AUTHORS replied that the only alcoholic solutions used had been in 95 per cent ethanol, and it would seem that the method could not be applied to 50 or 60 per cent ethanol solutions, because on heating there might be hydrolysis. There was a possibility of testing without heating since the reagent reacted readily with citral. A 1 cm. cell was used throughout and the quantities were such that one obtained suitable extinction reading on the spectrophotometer. They had no information on the age of the samples which Stafford Allen had sent, but the analyses were made about three weeks after they were made by the manufacturer. The amount of acacia involved was only 15 mg., heated for three minutes, so it would probably not interfere to any great extent. Reasonably anhydrous crystalline hydrazone gave values which compared favourably with those obtained from citral itself. The reagents had been recrystallised twice from ethanol and the constants obtained were identical. There was interference with the reaction when it was applied to injections such as progesterone in oil and with ethyl oleate the blanks were large. Ten variations had not produced a method which was successful in the presence of ethyl oleate.