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Biochem. J. (1964), **93**, 11

Determination of Oxo Steroids as Coloured Salts of their Girard Derivatives

BY E. MENINI* AND J. K. NORBYMBSKI†

M.R.C. Chemical Pathology of Steroids Research Unit, Jessop Hospital for Women, Sheffield

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A group of analytical methods is based on the observation that salts formed between strong acids and bases of relatively high molecular weight are extractable from aqueous solutions by chlorinated hydrocarbons (cf. Prudhomme, 1938; Auerbach,

1943; Lehman & Aitken, 1943; Jones, 1945; Barr, Oliver & Stubbings, 1948; Roy, 1956). In aqueous solution the equilibrium:

(A^+ and B^- being large ions, a^+ and b^- small ions) is shifted to the right by the extraction of A^+B^- with a chlorinated hydrocarbon. Consequently B^- may be extracted from its aqueous solution by the addi-

* Present address: M.R.C. Clinical Endocrinology Research Unit, 2 Forrest Road, Edinburgh.

† Present address: Laboratoire d'Endocrinologie, Université de Genève, Switzerland.

Table 1. *Colorimetric determination of carbonyl groups*

Experimental details are given in the text. The theoretical value for E_{410} was calculated as $E_1/E_2 \times n \times 100$, where E_1 is the extinction found, E_2 that expected per molar equivalent of the dye and n the number of reactive carbonyl groups.

Compound	Mol.wt.	No. of polar oxygen functions	No. of reactive carbonyl groups	E_{410} (% of theoretical)
Acetone	58	1	1	10
Cyclohexanone	98	1	1	50
Oestrone	270	2	1	87
Oestrone 3-methyl ether	284	1	1	87
Androst-4-ene-3,17-dione	286	2	2	82
5 α -Androstane-3,17-dione	288	2	2	82
Dehydroepiandrosterone	288	2	1	94
Testosterone	288	2	1	91
Androsterone	290	2	1	100
Androst-4-ene-3,11,17-trione	300	3	2	73
5 β -Androstane-3,11,17-trione	302	3	2	64
11-Oxoacetiocholanolone	304	3	1	94
11 β -Hydroxyacetiocholanolone	306	3	1	82
Pregesterone	314	2	2	86
Pregnenolone	316	2	1	93
5 β -Pregnane-3,20-dione	316	2	2	92
11-Oxoprogesterone	328	3	2	94
3-Oxobisnorchol-4-en-22-al	328	2	2	75
Cortisol	362	5	2	29
Tetrahydrocortisone	364	5	1	40
Cholest-4-en-3-one	384	1	1	72
5 α -Cholestanone	386	1	1	52

tion of a suitable A^+ . When A^+ is a dye and A^{b-} not extractable by the organic solvent the light-absorption of the extract provides a measure of B^- . Conversely, A^+ may be determined by the addition of a suitable B^- . Clearly, this analytical principle is applicable to such neutral compounds that can be converted into strong acids or bases in high yield. The present paper concerns the determination of oxo steroids by their conversion into quaternary ammonium bases

[Girard T derivatives:

followed by the formation of coloured salts of the latter with bromothymol blue (3',3''-dibromothymolsulphonphthalein).

Girard T derivatives were prepared in the usual manner (Girard & Sandulesco, 1936; for review see Wheeler, 1962). Conditions for their colorimetric determination were based on those reported by Auerbach (1943) with the arbitrary choice of bromothymol blue as the coloured anion and ethylene dichloride as solvent for extraction. The light-absorption of the yellow extract was determined at 410 m μ . Results recorded in Table 1 show that the efficiency of the method depends on molecular weight, number of reactive carbonyl groups and number of other polar oxygen functions. The low efficiency in the determination of 5 α -cholestan-3-one remains unexplained. 11-Oxo steroids as well as steroids lacking oxo groups failed to respond to the test. For dehydroepiandro-

Table 2. *Proportionality in the determination of dehydroepiandrosterone by its conversion into the Girard T derivative (bromothymol blue salt)*

Experimental details are given in the text. All values are means of duplicate determinations. $E_{615}/\mu g.$ was determined in alkaline solution (micro-method); $E_{410}/\mu g.$ was determined in ethylene dichloride solution (standard method).

Dehydroepiandrosterone ($\mu g.$)	$E_{615}/\mu g.$	Dehydroepiandrosterone ($\mu g.$)	$E_{410}/\mu g.$
0.28	0.146	2.8	0.0086
0.57	0.151	5.7	0.0093
0.85	0.184	8.5	0.0098
1.14	0.172	11.4	0.0104
1.42	0.171	17.1	0.0107
1.71	0.177	19.9	0.0108
2.28	0.185	22.8	0.0109

sterone Beer's law was obeyed within the range 2.5–25 $\mu g.$ (Table 2). The mean ($\pm$ s.d.) extinction of blanks (18 measurements) was 0.056 ± 0.003 . The corresponding value for dehydroepiandrosterone (2 $\mu g./ml.$ of final solution), derived from fourteen measurements corrected by subtraction of blanks, was 0.124 ± 0.003 . At this concentration the s.d. between duplicates was 0.004. Experimental conditions giving lower blanks were suitable for the assay of monoketones but led to a loss of efficiency with compounds with two reactive carbonyl groups.

In the presence of a trace amount of pyridine, bromothymol blue in ethylene dichloride exhibited

a spectrum ($\lambda_{\max}$, 410 $m\mu$, ϵ 19000) identical with those of the extracts obtained in the procedure outlined and presumably due to the quinonoid form of the dye (I, $X = OH$). Similarly, an alkaline solution of the dye exhibited a spectrum ($\lambda_{\max}$, 615 $m\mu$, ϵ 40000) identical with those of the alkali extracts of the ethylene dichloride solutions and presumably due to the quinonphenolate form of the dye (I, $X = O^-$; White & Acree, 1917). The higher extinction in alkali permits an increase in the sensitivity of the method by the introduction of an additional extraction step. Concurrent reduction of volume led to the determination of a mono-ketone within the range 0.3–2.3 μg . (Table 2) with good agreement between duplicate determinations (coefficient of variation 4 %).

After colorimetric measurement, the Girard cations were readily hydrolysed and the parent oxo steroids recovered in high yield.

The method outlined was adapted to the detection of oxo steroids on paper chromatograms (limit of detection 0.5–1.0 μg).

EXPERIMENTAL

Materials. Girard T reagent was recrystallized from ethanol and stored over KOH pellets in a desiccator. Ethylene dichloride was shaken vigorously and repeatedly with conc. H_2SO_4 until the latter remained colourless; the solvent was then washed successively with water, aq. KOH and water, dried with anhydrous Na_2SO_4 , refluxed over P_2O_5 and distilled. Acetic acid was refluxed over CrO_3 and distilled. Commercial bromothymol blue (British Drug Houses Ltd.) was used without further purification; repeated crystallization from various solvents (acetone–hexane, methanol or acetic acid) did not change its spectroscopic properties.

Standard method (measurement at 410 $m\mu$). A 1% (w/v) solution of Girard T reagent in acetic acid (0.1 ml.) and the carbonyl compound (usually 10–20 μg .) were left overnight at room temperature or heated at 100° for 20 min. and then diluted with 3.5 N- K_2CO_3 saturated with KCl (1.0 ml.) and with a saturated aqueous solution of bromothymol blue (2.0 ml.). After the addition of ethylene dichloride (6.0 ml.) the mixture was shaken slowly for 60 sec. and then centrifuged. The extinction of the ethylene dichloride solution was measured in cells of 10 mm. light-path at 410 $m\mu$. Reproducibility of results depended on the purity

of the ethylene dichloride and on adherence to the stated conditions of extraction.

Variation of conditions. (a) A number of determinations were performed by using 4 N- K_2CO_3 without addition of KCl: blanks were lower, and monohydroxy ketones of the androstane and pregnane series gave results closely comparable with those obtained by the standard method but more highly oxygenated ketones gave considerably lower values. (b) Washing [with aq. KCl (5–10 %, w/v)] of the ethylene dichloride extracts obtained by the standard method had the same effect as the use of 4 N- K_2CO_3 without the addition of KCl. (c) Methylene dichloride, chloroform and acetylene tetrachloride extracted the coloured salts; carbon tetrachloride, ether, benzene and toluene did not. (d) Results were not affected by varying the concentration of the Girard reagent (1–5 %, w/v) nor by the reaction time (10–240 min.) at 100°.

Micro-method (measurement at 615 $m\mu$). Dehydroepiandrosterone (0.25–2.5 μg .) was treated as in the standard method except that half the quantities of reagents and solvents were used. A sample of the ethylene dichloride extract (2.5 ml.) was washed with aq. 10% (w/v) KCl (1.0 ml.), and a sample of the organic phase (2.0 ml.) was extracted with 0.1 N- NaOH (0.5 ml.). The light-absorption of the alkaline solution was determined at 615 $m\mu$ in a micro-cell of 10 mm. light-path.

Recovery of oxo steroids after their measurement. Dehydroepiandrosterone (57.0 μg .) was determined by the standard method. The yellow ethylene dichloride solution was extracted with an equal volume of 0.1 N- NaOH , the blue extract made 2 N with respect to HCl and left for 2 hr. at room temperature. The mixture was extracted with ethylene dichloride (2 $\times$ 1 vol.), the extract washed with N- NaOH (2 $\times$ 0.2 vol.) and then with water (0.2 vol.), and evaporated to dryness. By using the standard method again, the residue was found to contain 96 % of the material originally estimated. Paper chromatography in system B_3 (Bush, 1952), namely light petroleum–benzene–methanol–water (67:33:80:20, by vol.), and in system E_4 (Eberlein & Bongiovanni, 1955), namely 2,2,4-trimethylpentane–2-methylpropan-2-ol–methanol–water (20:9:9:2, by vol.), revealed the presence of only one constituent, with the mobility of dehydroepiandrosterone. Five other compounds were similarly assayed, recovered and identified: androst-4-ene-3,17-dione (83 % recovery), 5 β -androstane-3,11,17-trione (70 %), 11 β -hydroxyaetiocholanolone (72 %), androst-4-ene-3,11,17-trione (84 %) and 3-oxobisnorchol-4-en-22-al (87 %). With androst-4-ene-3,17-dione the recovered material was estimated by measurement of its extinction at 240 $m\mu$.

Detection of oxo steroids on paper chromatograms. Dehydroepiandrosterone, androst-4-ene-3,17-dione and androst-4-ene-3,11,17-trione (0.5–3 μg . of each) were chromatographed in the system E_4 . The papers were treated with 1% (w/v) Girard T reagent in acetic acid and allowed to dry at room temperature (30–40 min.). They were then treated with a mixture of saturated aq. bromothymol blue, 4 N- K_2CO_3 and ethanol (2:1:1, by vol.). Both reagents were applied to paper by means of a trough and roller without causing the spots to migrate. After 3–5 min. each paper was placed in a trough and covered with ethylene dichloride; areas occupied by ketones appeared as white spots on a blue background. Quantities of 0.5–1 μg . were just detectable; 2–3 μg . gave very distinct spots.

DISCUSSION

The analytical method reported above is suited to the detection of all ketones and aldehydes that react with the Girard T reagent. Their measurement is, however, the less accurate the lower a compound's molecular weight and the greater its number of reactive carbonyl groups and other polar oxygen functions (hydroxyl groups and unreactive oxo groups). Under the conditions employed, compounds of the androstane and pregnane series with one or two reactive carbonyl groups and with altogether not more than three polar oxygen functions are determined with acceptable accuracy (see Table 1). In contradistinction to other methods commonly used for the determination of oxo steroids, the present assay does not distinguish between different types of oxo groups (e.g. $\alpha\beta$ -unsaturated ketones and saturated ketones) provided that they react with the Girard T reagent. Hence the assay may be used for the determination of the number of reactive carbonyl groups in a molecule. An attractive feature of the method is that oxo compounds, after having been determined, can be separated from other constituents of the analytical sample and recovered in good yield. The scope of the method is not limited to the determination of ketones and aldehydes: indirect determination of secondary alcohols by their initial oxidation to ketones (cf. Menini & Norzymski, 1962) and assessment of the efficiency of Girard separations are two obvious applications. Though we had no opportunity of applying the assay to biological material, Dr R. A. Harkness informs us that he had used it with success for the determination of 5β -pregnane-3,20-dione derived from the oxidation of urinary pregnanediol and pregnanolone.

SUMMARY

1. Salts formed between the Girard derivatives of carbonyl compounds and bromothymol blue were preferentially extracted from aqueous solution with ethylene dichloride. Colorimetric measurement of the extracted salts gave an accurate estimate of compounds of the androstane and pregnane series with one or two reactive carbonyl groups and altogether not more than three polar oxygen functions.

2. The method outlined was adapted to the detection of oxo steroids on paper chromatograms.

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Biochem. J. (1964), **93**, 14

The Isolation and Composition of Ribonucleic Acids from Leaves

By A. R. TRIM, JANET E. BAKER AND A. B. LEAH

Plant Breeding Institute, Maris Lane, Trumpington, Cambridge, and The Agricultural Research Council's Virus Research Unit, Huntingdon Road, Cambridge

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The most extensive investigation of the nucleotide composition of plant ribonucleic acids is that of Vanyushin & Belozersky (1959), who analysed the whole RNA of seed, pollen and thallus material from a wide taxonomic range of plants by using a modified Schmidt & Thannhauser (1945) method. The work was handicapped by the interference of

ultraviolet-absorbing substances other than nucleotides which are present in most plant tissues. Partly because of this difficulty a preliminary separation of the nucleic acids from the plant organs is desirable. In the present paper, following earlier attempts to obtain a quantitative recovery of minimally degraded nucleic acid from leaves