The Gas-Liquid Chromatography of Carboxylic Acid Esters of the Urinary 11-Deoxy-17-oxo Steroids

DETERMINATION AS *n*-BUTYRATES

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1. The gas-liquid-chromatographic separations of the acetate, propionate, *n*-butyrate, isobutyrate and *n*-valerate esters of androsterone, aetiocholanolone and dehydroepiandrosterone were studied on a 1% neopentyl glycol sebacate column. The *n*-butyrate, isobutyrate and *n*-valerate esters were well resolved.
2. The three steroids derived from hydrolysed urinary 17-oxo steroid conjugate extracts were analysed by gas-liquid chromatography after conversion into their *n*-butyrate esters. The results were compared with independent determinations involving chromatography on alumina.

Several workers have attempted to increase the speed of analysis of urinary 11-deoxy-17-oxo steroids by the application of gas-liquid chromatography (Haahti, VandenHeuvel & Horning, 1961; Sparagana, Keutmann & Mason, 1963). Unfortunately, no stationary phase has yet been found that separates dehydroepiandrosterone, androsterone and aetiocholanolone in their free forms. However, VandenHeuvel, Creech & Horning (1962) showed that the trimethylsilyl ethers of these steroids could be separated on a stationary phase of XE-60 (cyanoethylmethylsilicone), and Kirschner & Lipsett (1964) separated the trimethylsilyl ethers of dehydroepiandrosterone and epiandrosterone on a stationary phase of QF-1 (methyltrifluoropropylsilicone). Trimethylsilyl ethers have also been used by France, Rivera, McNiven & Dorfman (1965) and by Thomas (1965).

Lipsky & Landowne (1961) studied a number of stationary phases for the separation of steroid mixtures. One of the more satisfactory ones, with respect to thermal stability and resolving power, was neopentyl glycol sebacate. Although androsterone and aetiocholanolone were not resolved, their acetates showed slight separation, and resolution of the acetates of dehydroepiandrosterone and aetiocholanolone was greater than that of the corresponding free steroids. In the present work, a series of aliphatic esters of the 11-deoxy-17-oxo steroids were prepared to study the effect of size and length of the alkyl group on the resolution obtainable on a stationary phase of neopentyl glycol sebacate.

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MATERIALS AND METHODS

Reagents and solvents. Solvents other than ethanol (R.R. grade; James Burrough Ltd., London, S.E. 11) were of laboratory-reagent grade and were distilled before use. Pyridine was dried over KOH and distilled. *n*-Butyric anhydride and *n*-valeric anhydride were refluxed over CaC₂ and distilled. Other reagents were used as supplied.

Gas-liquid chromatography. A Perkin-Elmer Vapor Fractometer (model 451) fitted with a ⁹⁰Sr argon-ionization detector was used. Columns were stainless-steel U-tubes (1 m. × 4.5 mm. internal diam.) and were packed with Celite (Johns-Manville and Co. Ltd., London, S.E. 1; mesh 100-120) that had been acid-washed by the method of Horning, VandenHeuvel & Creech (1963) and coated with 1% neopentyl glycol sebacate by the technique of Zubyk & Conner (1960). Operating conditions were as follows; argon flow rate, 52-58 ml./min.; column temperature, 220°; injection-port temperature, 264°; detector voltage, -800 v. For 5 α -cholestane the column efficiency was 1500 theoretical plates.

Samples were introduced in chloroform solution (1-3 μ l.; 1 mg. of each component/100 μ l.) with a Hamilton syringe. Although this was a larger amount of material than is usually used with a ⁹⁰Sr argon-ionization detector and low-percentage stationary-phase columns, the steroid peaks were symmetrical, showing that the columns were not overloaded. For quantitative work, samples were mixed with a known weight of 17 β -hydroxy-5 α -androstan-3-one (stanolone) *n*-butyrate as an internal standard.

Gradient-elution analysis. This was carried out on alumina columns as described by Kellie & Wade (1957). The identities of the eluted components were established by paper chromatography on system A of Bush (1952).

Preparation of steroid esters. A solution of the steroid (100-200 μ g.) in pyridine (0.1 ml.) was treated with the appropriate anhydride (0.2 ml.) and kept at room temperature or 35° overnight. The reagents were removed in a

Table 1. *Preparation and properties of steroid esters*

Compound	Yield		Melting point	Analyses*	
	(mg.)	(%)		C (%)	H (%)
Dehydroepiandrosterone	51	83	121–122°	77.0	9.6
<i>n</i> -valerate				77.4	9.7
Aetiocholanolone <i>n</i> -valerate	Oil		—	—	—
Androsterone <i>n</i> -valerate	120	98	88.5–90	77.0	10.2
				77.0	10.2
Androsterone <i>n</i> -butyrate	231	91	104–105	76.7	9.9
				76.6	10.1
Aetiocholanolone <i>n</i> -butyrate	Oil				
Dehydroepiandrosterone	227	93	168–169	77.1	9.3
<i>n</i> -butyrate				77.0	9.6
Stanolone <i>n</i> -butyrate	170	67	88.0–88.5	76.4	9.7
				76.6	10.1

* Upper figures refer to found values, lower figures refer to calculated values.

stream of nitrogen. *n*-Butyrates and *n*-valerates were prepared on a larger scale (50–200 mg.) and recrystallized from aqueous methanol. Details are given in Table 1.

Preparation and analysis of urine extracts. 17-Oxo steroid conjugates were extracted from 24 hr. urine specimens by the method of Kellie & Wade (1957). The conjugate extract was hydrolysed with a limpet β -glucuronidase preparation (500 units/ml.) in acetate buffer, pH 4, overnight at 35°. The aqueous solution was then treated by the solvolysis procedure of Burstein & Lieberman (1958). The ethyl acetate extract, which contained the free 17-oxo steroids, was washed with *n*-NaOH (4 $\times$ 0.5 vol.) and then with water until the washings were neutral. The residue obtained by evaporating this extract to dryness was dissolved in a known volume of ethanol and a portion was assayed for total 17-oxo steroid content (Callow, Callow & Emmens, 1938). A portion containing about 1 mg. of total 17-oxo steroid was evaporated to dryness under nitrogen; a known weight of stanolone (about 0.5 mg.) was added and the mixture was esterified with *n*-butyric anhydride (0.2 ml.) and pyridine (0.1 ml.) overnight at 35° before gas-liquid-chromatographic analysis. A similar portion was analysed by gradient elution.

RESULTS AND DISCUSSION

Gas-liquid chromatography of the aliphatic esters of dehydroepiandrosterone, androsterone and aetiocholanolone. The effect of ester formation on the gas-liquid-chromatographic separation of dehydroepiandrosterone, androsterone and aetiocholanolone is shown in Fig. 1. The resolution and separation factor of each pair of adjacent peaks are given in Table 2. Resolution increases as the length of the side chain at C-3 increases. The isobutyrate of androsterone and aetiocholanolone show a resolution between those of the propionates and *n*-butyrates, whereas the isobutyrate of aetiocholanolone and dehydroepiandrosterone show resolution comparable with that of the *n*-valerates.

The ΔR_M values for the esters were determined from their retention times relative to 5 α -cholestane

Fig. 1. Separation of the carboxylic acid esters of androsterone, aetiocholanolone and dehydroepiandrosterone by gas-liquid chromatography on 1% neopentyl glycol sebacate. (a) Free steroids; (b) propionates; (c) *n*-valerates. A, Androsterone or ester; E, aetiocholanolone or ester; D, dehydroepiandrosterone or ester; C, 5 α -cholestane as internal standard. The column temperature was 220° and the column length 1 m.

(Knights & Thomas, 1962) and are given in Table 3 together with values for the esters of epiandrosterone. With the exception of the isobutyrate the

ΔR_M values for the esters of androsterone (3-axial) are lower than those for the esters of aetiocholanolone, dehydroepiandrosterone and epiandrosterone (3-equatorial).

The *n*-butyrate, isobutyrate and *n*-valerate esters of androsterone, aetiocholanolone and dehydroepiandrosterone were all well resolved. The *n*-butyrate ester derivatives were used for the determination of the three steroids in urine extracts. Gas-liquid-chromatographic analysis of small-scale preparations of the *n*-butyrates showed no sign of peaks corresponding to the free steroids, and the good yields obtained in the large-scale preparations of esters suggests that the derivatives are formed quantitatively. Although epiandrosterone *n*-butyrate had a slightly longer retention time than dehydroepiandrosterone *n*-butyrate, when chromatographed together the two compounds were not resolved.

Calibration graphs and analysis of synthetic mixtures. Calibration graphs were plotted from

Table 2. *Effect of increasing size of the aliphatic group on the separation and resolution of 17-oxo steroid esters*

Δ_{AB} etc., Resolution between androsterone and aetiocholanolone or their esters; S_{AB} etc., separation factor (Glueckauf, 1955) between androsterone and aetiocholanolone or their esters.

Grouping at C-3	Androsterone-aetiocholanolone		Aetiocholanolone-dehydroepiandrosterone	
	Δ_{AB}	S_{AB}	Δ_{ED}	S_{ED}
Hydroxy	No separation	1.00	0.98	1.06
Acetate	Small	1.06	1.01	1.12
Propionate	0.92	1.10	1.28	1.14
<i>n</i> -Butyrate	1.29	1.14	1.46	1.16
Isobutyrate	1.17	1.13	1.66	1.18
<i>n</i> -Valerate	1.79	1.20	1.61	1.18

standard solutions of the *n*-butyrates of androsterone, aetiocholanolone, and dehydroepiandrosterone by using stanolone *n*-butyrate as internal standard. As aetiocholanolone *n*-butyrate could not be crystallized, a known weight of aetiocholanolone was esterified with *n*-butyric anhydride and the resulting oil was made up to a standard volume with ethanol. Quantitative esterification of the steroid was assumed. Linear weight-ratio/area-ratio relationships were established for all three esters (Fig. 2) and androsterone *n*-butyrate and aetiocholanolone *n*-butyrate gave the same response. Peak area ratios did not vary for a given weight ratio when the absolute amount of steroid injected on to the column varied in the range 5–40 μ g. for each component.

Four mixtures containing known weights of androsterone, aetiocholanolone, dehydroepiandrosterone and stanolone were prepared and esterified, the products were dissolved in chloroform and portions were analysed by gas-liquid chromatography. That good agreement was obtained with the amounts known to be present confirmed that esterification of all four steroids was reasonably quantitative.

Comparison of gas-liquid-chromatographic and gradient-elution analysis. Hydrolysed conjugate extracts from nine urines were analysed by both methods. In addition, portions from urines nos. 1–4, to which stanolone had been added, were submitted to thin-layer chromatography on silica gel in the system ethyl acetate-cyclohexane (1:1 v/v), and in each case the area containing androsterone, aetiocholanolone, dehydroepiandrosterone and stanolone was eluted with ethanol. The dry residues thus obtained were esterified and analysed by gas-liquid chromatography. A comparison of the results obtained by these procedures is shown in Table 4.

Known weights of androsterone, aetiocholanolone and dehydroepiandrosterone were added to urines nos. 6–9 and portions were analysed by both

Table 3. *Relative retention times and ΔR_M values of steroid esters with aliphatic acids*

The relative retention times (RRT) are given relative to that of 5 α -cholestane (12.1 min.). ΔR_M represents a change in R_M , i.e. $\log 1(R_T - 1)$, due to a change in molecular structure other than a simple substitution of a hydrogen atom (Bush, 1961), in this instance a change in the aliphatic ester at C-3.

Grouping at C-3	Androsterone		Aetiocholanolone		Dehydroepiandrosterone		Epiandrosterone		Stanolone	
	RRT	ΔR_M	RRT	ΔR_M	RRT	ΔR_M	RRT	ΔR_M	RRT	ΔR_M
Hydroxy	1.60	—	1.60	—	1.87	—	1.87	—	2.14	—
Acetate	1.50	-0.03	1.57	-0.01	1.77	-0.02	1.92	0.01	—	—
Propionate	1.82	0.06	2.00	0.10	2.29	0.09	2.54	0.13	—	—
<i>n</i> -Butyrate	2.36	0.17	2.69	0.23	3.14	0.23	3.38	0.26	3.65	0.23
Isobutyrate	1.82	0.16	2.07	0.11	2.44	0.12	2.66	0.15	—	—
<i>n</i> -Valerate	3.15	0.29	3.79	0.38	4.46	0.38	4.27	0.36	—	—

gas-liquid chromatography and by gradient elution (Table 5). Fig. 3 shows a typical chromatogram obtained from a urine extract (urine no. 1). Any epiandrosterone should be estimated with dehydroepiandrosterone.

Good agreement was found between the results of gas-liquid-chromatographic and gradient-elution

Fig. 2. Calibration graph for gas-liquid chromatography of *n*-butyrates of androsterone (●), aetiocholanolone (○) and dehydroepiandrosterone (△). Experimental conditions were as given in Fig. 1.

analyses except with urine no. 2. Agreement was also found between the amounts of steroids added to urine and the increases estimated by gas-liquid chromatography. With urine no. 2, the gas-liquid-chromatographic method consistently gave lower values for aetiocholanolone and dehydroepiandrosterone than the gradient-elution method. The low values were not due to an impurity in the urine extract with same retention time as stanolone *n*-butyrate, since a chromatogram of an esterified portion of the extract to which no stanolone had been added showed no peak in this position. It appears that Zimmermann-positive chromogens were interfering with the gradient-elution analysis to give anomalously high results.

In the gradient-elution analyses, a red pigment was eluted from the alumina column coincident with dehydroepiandrosterone and this caused interference with the Zimmermann determination. When the colour was intense, the gradient-elution analysis gave higher values for dehydroepiandrosterone than did gas-liquid-chromatographic analysis, and in such extracts the latter method is more reliable.

Two disadvantages of the gas-liquid-chromatographic method became apparent during the work. Most of the urine extracts contained a late-eluting component with a retention time of about 2.5 hr. (Fig. 3). This component was not removed from the extract by preliminary thin-layer chromatography, and because of this the total analysis time was much greater than the time taken for the 17-oxo steroid *n*-butyrates to be eluted. The other disadvantage was that the recorder pen did not completely return to the base line between each of the 17-oxo steroid *n*-butyrate peaks, so that the attenuation could not

Table 4. *Analysis of urinary 17-oxo steroids: comparison of results by gradient elution from alumina with gas-liquid chromatography on 1% neopentyl glycol sebacate*

GE, Gradient elution; GLG, gas-liquid chromatography; TLC+GLC, thin-layer chromatography followed by gas-liquid chromatography.

Urine sample no.	Androsterone (mg.)			Aetiocholanolone (mg.)			Dehydroepiandrosterone (mg.)		
	GE	GLC	TLC+GLC	GE	GLC	TLC+GLC	GE	GLC	TLC+GLC
1	0.28	0.29	0.26	0.49	0.44	0.45	0.06	0.05	0.06
2	0.14	0.15	0.14	0.48	0.28	0.29	0.55	0.40	0.40
2 (repeat)	0.22	0.13	0.16	0.42	0.31	0.32	0.52	0.44	0.44
3	0.08	0.06	0.04	0.45	0.39	0.33	0.12	0.06	0.05
4	0.16	0.17	0.16	0.12	0.10	0.10	0.80	0.74	0.69
5	0.42	0.44	—	0.16	0.21	—	0.09	0.07	—
6	0.15	0.12	—	0.43	0.40	—	0.09	0.07	—
7	0.53	0.50	—	0.24	0.26	—	0.04	Small	—
8	0.27	0.25	—	0.46	0.60	—	0.11	0.06	—
9	0.11	0.11	—	0.58	0.51	—	0.15	Small	—

Table 5. *Analysis of urinary 17-oxo steroids: comparison of results by gradient elution from alumina and gas-liquid chromatography with added 17-oxo steroids*

GE, Gradient elution; GLC, gas-liquid chromatography.

Urine sample no.	Androsterone (mg.)			Aetiocholanolone (mg.)			Dehydroepiandrosterone (mg.)		
	Added	Recovered		Added	Recovered		Added	Recovered	
		By GE	By GLC		By GE	By GLC		By GE	By GLC
6	0.40	0.37	0.39	0.08	0.05	0.13	0.20	0.20	0.21
7	0.20	0.20	0.18	0.16	0.17	0.20	0.20	0.17	0.21
8	0.24	0.21	0.25	0.00	-0.02	-0.05	0.40	0.42	0.45
9	0.32	0.30	0.32	0.12	0.13	0.18	0.32	0.39	0.43

Fig. 3. Gas-liquid-chromatographic analysis of urine no. 1. A, Androsterone *n*-butyrate; E, aetiocholanolone *n*-butyrate; D, dehydroepiandrosterone *n*-butyrate; S, stanolone *n*-butyrate (internal standard). Experimental conditions were as given in Fig. 1.

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be altered to increase the peak size of one component present in low concentration relative to the other two. This difficulty could be overcome if more efficient columns were used. Butyrate esters are stable derivatives whereas trimethylsilyl ethers are very sensitive to traces of moisture and easily decompose to the parent steroid. However, the retention times of the butyrate esters are longer than those of the corresponding trimethylsilyl ethers when chromatographed under similar conditions.