Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 443–450. Pergamon Press. Printed in Great Britain

MASS SPECTROMETRIC IDENTIFICATION OF TESTOSTERONE,* ANDROSTENEDIONE, DEHYDROEPIANDROSTERONE, DIHYDROTESTOSTERONE, AND ANDROSTANEDIOL IN HUMAN PERIPHERAL PLASMA

VENKATASESHU K. GANJAM† and BEVERLEY E. PEARSON MURPHY‡
Queen Mary Veterans Hospital and Department of Experimental Medicine, McGill University, Montreal, Canada

and

TAK-HANG CHAN and PETER A. CURRIE
Department of Chemistry, McGill University, Montreal, Canada

(*Received* 15 *February* 1973)

SUMMARY

An alkali-washed diethyl ether extract of 1300 ml of pooled adult human plasma was fractionated by passage through a single column of Sephadex LH-20. Without further purification the various fractions were subjected to mass spectrometric analysis. The presence of 17β-hydroxy-4-androsten-3-one, 4-androstene-3,17-dione, 3β-hydroxy-5-androsten-17-one, 17β-hydroxy-5α-androstan-3-one and 5α-androstane-3 (α or β), 17β-diol was noted. Since testosterone and DHEA occurred together with cholestadiene as did androstenedione with cholesterol, further separation was carried out by t.l.c. to confirm the identifications individually.

INTRODUCTION

THE RECENT development and application of refined chromatographic, isotopic and spectroscopic techniques permit accurate measurement of submicrogram amounts of hormones in biological fluids. The specific binding properties of certain proteins have been used to provide competitive protein-binding (CPB) assays for many hormones[1]. Investigation of the specificity of the testosterone-binding β-globulin or sex hormone-binding globulin (SHBG) of human serum has revealed that the only substances that bind to this globulin are other potent androgens and 17β-estradiol[2]. These steroids can be separated by Sephadex LH-20[3]. To confirm that these substances are eluted unchanged from the Sephadex columns, the substances eluted from pooled human plasma were examined by mass spectrometry. A preliminary report has appeared[4].

*The trivial names used in this study are: Testosterone = 17β-Hydroxy-4-androsten-3-one (T), Androstenedione = 4-Androstene-3,17-dione (Δ4-A); Dehydroepiandrosterone = 3β-Hydroxy-5-androsten-17-one (DHEA); Dihydrotestosterone = 17β-Hydroxy-5α-androstan-3-one (DHT); Androstanediol = 5α-Androstane-3β,17β-diol; Androsterone = 3α-Hydroxy-5α-androstan-17-one.

†Present address: Assistant Professor, University of Pennsylvania, School of Veterinary Medicine, New Bolton Centre, Kennett Square, Rd. # 2, Pa. 19348, U.S.A.

‡Associate, Medical Research Council of Canada.

EXPERIMENTAL

Materials

All reagents used were redistilled once. The non-radioactive steroid standard were recrystallized to constant m.p. and their purity checked by I.R. analysis. The tritiated standards, namely [1,2-^{3}H]-4-androstene-3,17-dione (S.A. 5·9 Ci/mmol), [1,2-^{3}H]-17β-hydroxy-5α-androstan-3-one (S.A. 44 Ci/mmol) and [1,2-^{3}H]-17β-hydroxy-4-androsten-3-one (S.A. 46 Ci/mmol) were obtained from New England Nuclear and purified once by thin layer chromatography (t.l.c.) (chloroform–methanol, 98:21, v/v); the chromatographic mobility of the purified radioactive standards was checked with that of the authentic, recrystallized standards. Thin-layer chromatography was performed using Silica gel GF-254 (E. Merck AG. Darmstadt, Germany) prewashed with nanograde methanol, heated to 800°C and cooled to room temperature. The slurry was made with double-distilled deionized water on glass plates pre-washed in nanograde methanol. After preparation the plates were re-run in nanograde methanol. Sephadex LH-20 was obtained from Pharmacia Ltd., Montreal, and was used as such. The column used (43 × 2·1 cm) was thoroughly washed and rinsed with nanograde chloroform (Mallinkrodt) as were the tubes in which various eluate fractions were collected. Pooled human plasma from both men and women, with sodium citrate added as anticoagulant, was obtained from the Red Cross, Montreal. The scintillation fiuid used consisted of 77 g of diphenyloxazole p/r 1 of toluene. Two or more hr after adding 10 ml scintillator, the samples were counted in a Phillips Liquid Scintillation Spectrometer.

Mass spectrometric analysis

All mass spectra were recorded on an AEI MS-902 mass spectrometer. The samples were introduced by a direct insertion probe at a temperature between 160–190°C (above ambient). The operating conditions were a 70 eV electron energy, resolution of 1000 and 8 kV accelerating voltage. the relative peak intensities were estimated by measurement of the heights of the peaks on the galvanometer recorder chart with a millimeter ruler. The mass number of each ion was established by counting.

Sephadex LH-20 chromatography

The purified tritiated standards of Δ^4-A, DHT and T (about 1·0 μCi in ethanol, with a mass of about 3·8 ng of each) were added to 1300 ml of pooled human plasma. After 30 min, the plasma was extracted 3 times with $1\frac{1}{2}$ vol of diethyl ether. The pooled extract was washed with 0·1 M NaOH to remove estrogens and the bulk of pigments and adjusted to pH 7·0 by several washes with equal vol of glass-distilled water. After evaporation to dryness *in vacuo*, the residue was dissolved in 1·0 ml of a solvent system consisting of heptane: chloroform–ethanol–water (50:50:1 by vol, water to incipient turbidity), applied to the column and eluted with the same solvent.

Two hundred fractions of 5·0 ml vol were collected, a 1·0 ml aliquot of each fraction was transferred to a scintillation vial, evaporated to dryness and counted for radioactivity following addition of 10 ml of scintillation fluid. The eluate fractions were then pooled according to the elution pattern of the labelled steroids, and subjected without further purification to mass spectrometric analysis.

RESULTS

Table 1 summarizes the Sephadex chromatographic fractionation.

Androstenedione

Fraction 1 contained, according to its mass spectrum, a mixture of androstenedione and cholesterol. The fraction was therefore further separated by t.l.c. (benzene–ethyl acetate, 4:1). The area corresponding to the R_F of Δ^4-A was again identified by comparison with authentic standard and was eluted by partition between benzene and water. The benzene layer was dried and the residue was analyzed by mass spectrometry. The mass spectrum obtained was closely similar

Table 1. Description of the various eluate fractions

Elution volume subjected to m.s.	Fraction #	Tritiated standard
60–85 ml	1	Androstenedione
135–155 ml	2	Dihydrotestosterone
170–205 ml	3	Testosterone
215–260 ml	4	—
265–310 ml	5	—
316–360 ml	6	—
365–410 ml	7	—
415–460 ml	8	—
465–500 ml	9	—
510–580 ml	10	—
580–600 ml	11	—
600–700 ml	12	—
700–800 ml	13	—
800–900 ml	14	—
900–1000 ml	15	—

Table 2. Androstenedione

m/e	% Relative intensities	
	Authentic standard	Plasma fraction #1*
288	6	7
287	25	24
286 (M+)	100	100
271	11	11
244	55	59
201	21	29
185	39	19
150	25	24
149	28	36
148	47	56
129	32	40
124	89	93
109	42	64
107	47	80
105	46	56
97	57	48
91	47	59

*Human plasma fraction #1 following t.l.c. separation.

to that of an authentic sample of Δ^4-A (Table 2). The ions in the high mass region showed a shift of two mass units when compared to the mass spectrum of T indicating that the fragmentation patterns were the same. the ion at $m/e = 124$ is common to both Δ^4-A and T and is due to the fragment derived from ring A (vide infra).

Table 3. Dihydrotestosterone

m/e	% Relative intensities	
	Authentic standard	Plasma fraction #2
292	5	8
291	30	28
290	86	88
275	8	8
272	10	12
257	10	10
246	19	21
233	11	15
232	15	18
231	100	100
215	13	15
199	12	18
163	38	32
144	21	18
135	19	18
124	32	30
123	45	40
121	19	20
109	30	28
107	36	30
105	24	20

Table 4. Testosterone

m/e	% Relative intensities	
	Authentic standard	Plasma fraction #3*
290	2	5
289	12	18
288 (M+)	45	57
273	6	8
270	5	8
247	21	24
246	37	40
228	12	16
203	20	27
185	6	9
165	12	14
147	30	46
124	100	100
123	11	13
109	23	37
107	9	11
105	18	27

*Human plasma fraction #3, following t.l.c. separation corresponding to testosterone standard on the thin-layer plate.

Dihydrotestosterone

Fraction 2 which contained the bulk of the [^{3}H]-DHT showed a mass spectrum which is identical to that of the standard DHT (Table 3). The electron impact-induced fragmentation reactions of 17β-hydroxysteroids have recently been studied by Spiteller–Friedmann and Spiteller [5]. The major fragment ion of DHT was found to be the rupture of the C-13/C-17 and C-14/C-15 bonds with the loss of C_3H_7O to give the base peak at $m/e = 231$. This is in agreement with our observations. 17β-Hydroxy-5β-androstane-3-one exhibited distinctly different mass spectral behavior and can thus be excluded from structural consideration [5].

Testosterone and dehydroepiandrosterone

The Sephadex LH-20 system failed to separate DHEA from T; in fact, the mass spectrum of fraction 3 showed that it consisted of a mixture of cholestadiene, T and DHEA. Fraction 3 was therefore subjected to further separation by t.l.c. (chloroform–methanol, 98:2, vv). With authentic standards running in parallel as references, separation of T and DHEA was achieved with R_Fs 0·25 and 0·40 respectively. Each individual fraction was eluted and the mass spectrometry was repeated. No interfering peaks from the t.l.c. extracts were observed. The fraction with R_F 0·25 showed mass spectral data identical in all respects with that of an authentic sample of T (Table 4). The mass spectrum of T has recently been reported by Spiteller [5]. The fraction with R_F 0·40 showed mass spectral data identical to that of an authentic sample of DHEA (Table 5). The fragmentation pattern of this compound has been briefly examined [6] and it can be readily interpreted. In addition to the loss of methyl group or water, which give rise to the M-15, M-18 peaks, the characteristic fragment ions are $m/e = 203$ and $m/e = 119$.

Table 5. Dehydroepiandrosterone

m/e	% Relative intensities	
	Authentic standard	Plasma fraction #3*
290	2	7
289	21	26
288 (M+)	100	100
273	9	12
271	12	17
270	59	53
256	14	13
255	65	62
213	23	30
203	56	56
177	28	28
159	35	36
145	40	44
135	19	29
133	23	25
131	22	24
121	33	50
119	30	39
107	59	69
105	46	54

*Human plasma fraction #3 following t.l.c. separation corresponding to DHEA standard on the thin-layer plate.

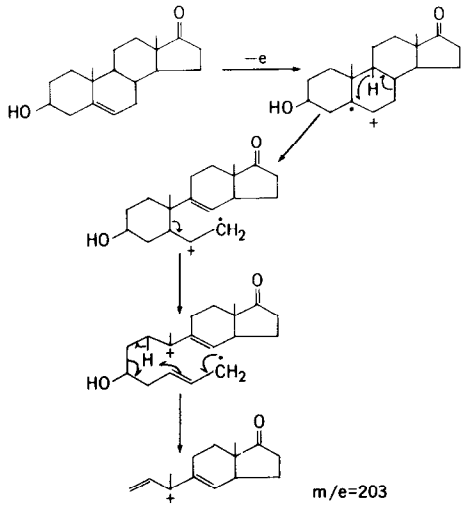

Fig. 1. Fragmentation pattern of dehydroepiandrosterone.

Table 6. Androstanediol

m/e	% Relative intensities	
	Authentic standard	Plasma fraction #10
294	5	7
293	22	24
292 (M+)	100	100
277	24	27
274	20	24
259	13	25
256	4	4
248	17	19
234	16	21
233	30	33
217	26	25
215	27	30
201	6	7
166	16	15
165	20	23
150	5	4
147	9	8
135	16	17
123	39	38
109	36	37
107	25	23

The origin of the ion $m/e = 119$ was ascribed to the rupture of ring B [6]. The ion of $m/e = 203$ is most likely to be due to fragmentation of the type shown in Fig. 1.

Androstanediol

Fraction 10 from the Sephadex column showed mass spectral data similar to that of standard 5α-androstane-3β,17-diol (Table 6). The mass spectra of androstanediols have recently been studied [5] and the fragmentation pattern was found to be influenced strongly by the 17-hydroxy group. The 5β-isomers showed in

addition, however, much enhanced M-18 fragmentation and can be differentiated easily from 5α-compounds. The mass spectrum of 5α-androstane-3α, 17β-diol is only slightly different from that of the 3β-isomer [5] and thus the structural assignment to fraction 10 cannot be made on the basis of mass spectral data alone. On the other hand, when the material eluted from the Sephadex column was assayed by competitive protein-binding and the position compared with authentic 3α- and 3β-isomers run in parallel, the results indicated that the compound has more likely the 3α-structure [3].

DISCUSSION

The presence of testosterone [6, 7], androstenedione [8, 9] and dehydroepiandrosterone [10] in human peripheral plasma has been well documented. Our study provides further confirmation of the identity of these steroids in plasma and confirms the validity of the LH-20 separation procedure. It also confirms unequivocally the presence of DHT and androstanediol(s).

In 1968, on the basis of binding studies with SHBG, we suggested that some 17β-hydroxysteroids other than T and DHT might account for part of the androgenic material in plasma [2]. No previous reports of androstanediol in blood have appeared although its presence was suggested by the work of Mauvais–Jarvis *et al.* [11] who isolated it in urine. The results of their study suggested that the 3α-hydroxy isomer is predominant. Since the 5α-androstanediols are bound very strongly to SHBG it has been possible to quantitate them in plasma [13]. While the levels are low compared to those of testosterone in men, they are relatively high in women, especially during pregnancy.

Even though androstenediol (5-androstene-3β,17β-diol) has been reported to be naturally existing in biological media [14], we failed to detect its presence to any significant extent in the present study. Although a peak corresponding to this steroid was found in all the sera we have fractionated to date [13] none was found in blood bank material (which is routinely treated with sodium citrate as an anticoagulant). Since androstenediol appears to be more labile than the other steroids, this discrepancy can probably be attributed to breakdown either due to the anticoagulant or to the conditions of storage.

Finally, we would like to point out the usefulness of Sephadex LH-20 column chromatography as a separation technique in steroid studies. Compared to paper and thin-layer chromatography, it has the considerable advantage of freedom from blank problems [3]. Another important advantage of this type of chromatography exemplified in this report is the large amount of material which can be readily processed. As described here, even a single chromatography may effect sufficient purification to permit identification of the material by mass spectrometry.

REFERENCES

1. Murphy B. E. P.: *Nature* **201** (1964) 679.
2. Murphy B. E. P.: *Recent Progress in Hormone Research* (Edited by E. B. Astwood). Academic Press **25** (1969) 563.
3. Murphy B. E. P.: *Acta endocr., Copenh.* **64** (1970) 37.
4. Ganjam V. K., Currie P. A., Chan T. H. and Murphy B. E. P.: *J. steroid Biochem.* **2** (1971) 155.
5. Spiteller-Friedmann M. and Spiteller G.: *Organic mass Spectrometry* **1** (1968) 231.
6. Friedland S. S., Lane G. H., Longman R. T., Train K. E. and O'Neal M. J.: *Analyt. Chem.* **31** (1959) 169.

7. Oertel G. W. and Eik-Nes K.: *Proc. Soc. exp. Biol. Med.* **102** (1959) 553.
8. Hollander N. and Hollander V. P.: *J. clin. Endocr. Metab.* **18** (1958) 1966.
9. Gandy H. M. and Peterson R. E. (Abstract). Program 46th Meeting. American Endocrine Society, San Francisco 1964.
10. Horton R. and Tait J. F.: *Proc. 2nd Int. Congr. Endoc., London* 1964. *Excerpta med. int. Congr. Ser.* **83** (1965) 262.
11. Gupta D.: *Steroids* **10** (1967) 457.
12. Mauvais-Jarvis P., Guillemont S., Corvol P., Floch H. H. and Bardow L.: *Steroids* **16** (1970) 173.
13. Murphy B. E. P.: *Principles of Competitive Protein-Binding Assays* (Edited by W. D. Odell and W. H. Daughaday). J. B. Lippincott (1971).
14. Laatikainin T., Laitinen E. A. and Vihko R.: *J. clin. Endocr. Metab.* **33** (1971) **59**.