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CAPILLARY GAS CHROMATOGRAPHY AS A TOOL FOR CHARACTERIZATION OF URINARY STEROID EXCRETION IN PATIENTS WITH CONGENITAL ADRENAL HYPERPLASIA

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Summary—Urinary steroid excretion was studied by capillary gas chromatography in 23 patients with congenital adrenal hyperplasia. In 5 patients the estimated excretion rates of pregnanetriol were in or below the normal range and 7 patients presented supranormal excretion rates of tetrahydro-cortisone and/or other glucocorticoid metabolites. Deficiency of 21-hydroxylase was nevertheless demonstrated in each patient by an increased ratio of excreted precursors vs products of 21-hydroxylase, e.g. of pregnanetriol/tetrahydro-cortisone. Due to this relative deficiency of glucocorticoids the patients' steroid excretion was further characterized by a predominance of 5α-hydrogenated $C_{19}O_3$ metabolites (11-keto-androsterone, 11-hydroxy-androsterone) over their 5β-hydrogenated homologues (11-keto-etiocholanolone, 11-hydroxy-etiocholanolone). An apparent preponderance in the excretion of pregnenetriol over that of pregnanetriol was found in 4 patients, but the presence of pregnenetriol was not confirmed by mass spectrometry following prepurification of the urine samples by thin-layer chromatography indicating interference of an unidentified steroid metabolite with the initial gas chromatographic analysis. The simultaneous determination of steroids serving as precursors or products of 21-hydroxylase by capillary gas chromatography helps to establish the diagnosis of 21-hydroxylase deficiency and to characterize the pattern of steroid excretion in this syndrome even in patients where the estimation of single urinary steroids may lead to erroneous conclusions.

INTRODUCTION

Determination of both plasma and urinary steroid concentrations is used to establish the biochemical diagnosis in patients with congenital adrenal hyperplasia (CAH). Radioimmunoassays for the estimation of several plasma steroids are readily available, but the limited specificity of the respective antibodies necessitates prepurification procedures which in turn limit the number of samples processed at once. Moreover, the interpretation of single determinations of plasma steroid concentrations is hampered by the episodic secretion of steroid hormones. These problems are avoided by the simultaneous determination of urinary steroids and steroid metabolites by means of capillary gas chromatography (GC) which integrates the area under the concentration vs time curves and offers the possibility to describe the relative part of excreted steroids characteristic for a specific clinical situation [1–5]. In the present study capillary gas chromatography was used to characterize the steroid excretion pattern of 23 patients with CAH.

EXPERIMENTAL

Patients

Twenty-three patients with CAH, aged 4–50 years, without any treatment or untreated for at least 3 days were included in the study. The aim of the investigation was explained to each patient and/or his/her parents and informed consent was obtained in each case. The diagnosis had been established in each patient by family history and by personal history (prenatal virilization or progressive virilization in early childhood, salt wasting crisis), by advanced bone age and by biochemical criteria (i.e. elevated plasma concentrations of 17-hydroxy-progesterone and/or increased excretion rates of pregnanetriol). Those patients who had received glucocorticoid therapy prior to the onset of the study were told to discontinue medication for 3 days on an outpatient basis. Subsequently a 24-h urine was collected from each patient and glucocorticoid therapy was reinstituted. Five healthy women, aged 19–27 years, who had not taken oral contraceptives for at least 6 months and 5 healthy men, aged 21–28 years served as controls. In healthy women steroid excretion was determined between the 5th and the 7th day of their menstrual cycle.

Reference steroids were purchased from Steraloids (Wilton, NH, U.S.A.) and from Makor Chemical (Jerusalem, Israel) and purity was checked by controlling melting point in each case.

Urine processing [1]

Urine samples (5 ml) of 24 h collections were

acidified to pH 5.2 by addition of 0.2 N HCl. β-Glucuronidase/aryl sulfatase (20,000 FU, Calbiochem, San Diego, CA, U.S.A.) was added, the hydrolysis taking place at 37 C. Hydrolysis did not affect pH values. After a total hydrolysis period of 24 h, 50,000 dpm [^{3}H]cortisol (NET 396, New England Nuclear, Boston, MA, U.S.A. sp. act. 115 Ci/mmol) were added to estimate recovery. The unconjugated steroids were extracted with 3 times 20 ml ethylacetate and the combined organic extract was washed once with 20 ml of 0.1 N NaOH and at least twice with 20 ml of double distilled water until neutrality was reached. Anhydrous sodium sulfate (5–10 g) was then added to each sample. After 12 h the solvent was distilled off at 35°C at reduced pressure. The residue was transferred with 3 × 1 ml ethylacetate into a centrifuging tube and the solvent was evaporated in a stream of dry nitrogen at 35°C.

Derivatisation [3, 6]

Samples were dissolved in 100 µl of a solution of methoxyamine hydrochloride (2% in pyridine; Pierce, Rockford, IL, U.S.A.) and heated at 80 C for 60 min. After evaporation with dry N_2 at 30 C 100 µl of a mixture of trimethylsilylimidazole, *N,O-bis*(trimethylsilyl)-acetamide and trimethylchlorosilane (3:3:2, TRI-SIL "TBT", Pierce) was added and the mixture was heated to 60°C for 20 h. After evaporation to dryness by dry N_2 (25°C, overnight) 2 ml of dichloromethane and 1.5 ml of 0.1 N H_2SO_4 were added to each sample, the mixture was shaken and the aqueous (lighter) phase was pipetted

Table 1A. Gas chromatographic characteristics of derivatized standard steroid compounds

No.	Steroid	Trivial name	Methylene units (as MO–TMS derivatives)
1		*n*-C_{24}-Alkane	24.00
2	5α-Androstane-3α-ol-17-one	Androsterone (An)	25.09
3	5β-Androstane-3α-ol-17-one	Etiocholanolone (Et)	25.28
4	5-Androstene-3β-ol-17-one	Dehydroepiandrosterone (DHEA)	25.68
5	5α-Androstane-3β-ol-17-one		25.83
6	5-Androstene-3β,17β-diol		25.92
7	5α-Androstane-3α-ol,11,17-dione	11-Ketoandrosterone (11 O-An)	26.01
8	1,3,5 (10)-Estratriene-3-ol-17-one		26.01
9	5β-Androstane-3α-ol-11,17-dione	11-Ketoetiocholanolone (11 O-Et)	26.14
10	4-Androstene-3,17-dione		26.19/26.24
11	1,3,5 (10)-Estratriene-3,17β-diol		26.38
12	4-Androstene-17β-ol-3-one		26.47
13	5β-Pregnane-3α-ol-20-one		26.87
14	5α-Androstane-3α-11β-diol-17-one	11β-Hydroxyandrosterone (11 OH-An)	26.96
15	5β-Androstane-3α-11β-diol-17-one	11β-Hydroxyetiocholanolone (11 OH-Et)	27.13
16	5β-Pregnane-3β-ol-20-one		27.15
17	5α-Pregnane-3α,20α-diol		27.42
18	5β-Pregnane-3α,20α-diol		27.62
19	5-Pregnene-3β-ol-20-one		27.67
20	5β-Pregnane-3α,17α,20α-triol	Pregnanetriol (PT)	27.95
21	5-Androstene-3β-16α,17α-triol		28.16
22	4-Pregnene-3,20-dione		28.20/28.25
23	5-Androstene-3β,16α,17β-triol		28.41
24	5β-Pregnane-3α,17α,21-triol-20-one		28.67
25	5β-Pregnane-3α,21-diol-20-one		28.76
26	1,3,5 (10)-Extratriene-3,16α,17β-triol		28.76
27	5α-Pregnane-3α,21-diol-20-one		28.91
28	5α-Pregnane-3α,17α,21-triol-20-one		29.02
29	5β-Pregnane-3α,17α,20-triol-11-one	Pregnanetriolone (PT'on)	29.02
30	5-Pregnene-3β,17α,20-triol	Pregnenetriol (PeT)	29.47
31	5β-Pregnane-3α,17α,21-triol-11,20-dione	Tetrahydro-Cortisone (TH-E)	29.66
32	5β-Pregnane-3α,21-diol,11,20-dione		29.75
33	5β-Pregnane-3α,11β,21-triol-20-one		30.00
34	5α-Pregnane-3α,11β,21-triol-20-one		30.12
35	5α-Pregnane-3α,17α,21-triol-11,20-dione	Allotetrahydro-Cortisone (allo-TH-E)	30.25
36	5β-Pregnane-3α,11β,17α,21-tetrol-20-one	Tetrahydro-Cortisol (TH-F)	30.27
37	5α-Pregnane-3α,11β,17α,21-tetrol-20-one	Allotetrahydro-Cortisol (allo TH-F)	30.37
38	5β-Pregnane-3α,17α,20α,21-tetrol-11-one		30.49
39	5β-Pregnane-3α,17α,20β,21-tetrol-11-one		30.77
40	5β-Pregnane-3α,11β,17α,20β,21-pentol		30.77
41	5-Cholesten-3β-ol		30.89
42	5β-Pregnane-3α,11β,17α,20α,21-pentol		31.22
43		*n*-C_{32}-Alkane	32.00

Table 1B. Trivial names of other steroids mentioned in this paper:

4-Androstene-11β-ol-3,17-dione	11β-Hydroxyandrostenedione (11 OH-Δ)
4-Androstene-3,11,17-trione	11-Ketoandrostenedione (11 O-Δ)
4-Pregnene-11β,17α-diol-3,20-dione	21-Desoxycortisol
4-Pregnene-17α-ol-3,20-dione	17α-Hydroxyprogesterone (17 OH-P)
4-Pregnene-17α,21-diol-3,11,20-trione	Cortisone
4-Pregnene-11β,17α,21-triol-3,20-dione	Cortisol

Trivial names are given for metabolites quantified in patients with congenital adrenal hyperplasia.

off. The organic layer was washed once with 1 ml of double distilled water. If the pH of the washing was above 7, the organic layer was then diluted with 1 ml dichloromethane and washed with water until the washings were neutral. Anhydrous sodium sulfate was added to the organic solutions and the samples were transferred into reactivials; the solvent was evaporated with dry N_2 at room temperature and the residue was dissolved in 100 μl of a mixture of ethylacetate-*n*-hexane (9:1 v/v) containing 100 ng/μl of alkanes *n*-$C_{24}H_{50}$ and *n*-$C_{32}H_{66}$. The 41 reference steroid compounds listed in Table 1 were derivatized in an identical fashion.

Recovery

Twenty μl of each sample were measured in a β-counter (Packard Instruments, La Grange, IL, U.S.A.). Mean recovery ($n = 75$) was $55.7 \pm 5.5\%$.

Gas chromatography

Gas chromatography was carried out using a Packard Instruments 428 gaschromatograph equipped with a 50 m OV 101 wall coated open tubular column [0.25 mm i.d., film thickness 0.25 μm, pretested at 180,000 effective plates by application of *n*-$C_{14}H_{30}$ alkane (k′ = 5.4)] and flame ionization detector. Derivatized solution (1 μl) was applied on a solid injection device (Packard Instruments). N_2 was used as a carrier gas with a flow rate of approx. 0.8 ml/min. Following sample injection (300°C) the temperature was immediately programmed between 180 and 265°C at 0.4°C/min followed by an isothermal run at end temperature.

Steroid identification

Steroids were identified by comparison of their retention times expressed as methylene units (MU) and determined by interpolation between the two *n*-alkanes (*n*-$C_{24}H_{50}$ and *n*-$C_{32}H_{66}$). Trivial names of gas chromatographically determined steroid metabolites and of the other steroids mentioned in this paper are given in Table 1.

Thin-layer chromatography

In 4 urine samples, in which initial gas chromatographic analysis had suggested a preponderance of excreted pregnenetriol over pregnanetriol, derivatisation of the urine samples was repeated after isolation of pregnanetriol, pregnenetriol and pregnanetriolone had been achieved by thin-layer chromatography (cyclohexane–ethylacetate, 1:99).

Mass spectrometry

Positive identification of tetrahydro-cortisone (TH-E) in derivatized samples and of pregnanetriol, pregnenetriol and pregnanetriolone in prepurified, derivatized samples was sought by additional gas chromatographic–mass spectrometric (GC–MS) analysis using a Hewlett-Packard 5995 GC–MS system equipped with a 25 m fused silica capillary column coated with OV 101 stationary phase. The GC injector was maintained at 300°C. For the identification of pregnanetriol, pregnenetriol and pregnanetriolone the oven temperature was programmed from 180 to 260°C at a rate of 1°C/min, whereas a rise in the oven temperature from 200 to 264°C at a rate of 0.5°C/min was programmed for the identification of TH-E. Temperatures of the analyzer, introduction line and ion source were kept at 180, 280 and 150°C, respectively, and the electron energy was 70 eV. Identification of steroids was achieved by recording the total ion current scanning from 100 to 700 mass units at a scan speed of 380 amu/s (4 samples per 0.1 amu). To increase sensitivity for the determination of TH-E selected ion monitoring (SIM) was also used, the instrument having been set to monitor the ions at *m/e* 609.5 (M^+), 578.6 ($M^+ - 31$), 488.5 ($M^+ - 121$) and 398.5 ($M^+ - 211$).

Calculation of steroid excretion

An automatic integrator (Hewlett Packard 3385 A) was used for the calculation of the peak areas plotted during gas chromatographic runs. Response factors for the 2 *n*-alkanes ("f C_{24}" and "f C_{32}") and for each reference steroid standard ("f St") were determined by their injection in varying amounts. These response factors were checked and adjusted regularly (i.e. after every 15th injection of biological samples) by derivatizing and injecting defined mixtures of the reference compounds. Response factors for the two *n*-alkanes were practically unchanged (f C_{24}: 1.05 ± 0.18, f C_{32}: 1.07 ± 0.20) over the complete series of injections. It was unnecessary to introduce additional correction factors for the increasing elution temperatures. The excreted amounts of individual steroids were calculated by the following formula:

$$\text{mg/24 h} = \text{Area} \times \text{f}\,C_{24} \times \text{f St} \times 100/\text{Recovery (\%)} \times \text{Dilution}$$

Area: measured peak area

f C_{24}: response factor for hydrocarbon C_{24}; provides for the actually injected amount.

f St: response factor for individual steroid

Recovery: provides for the yield of the complete treatment of the samples prior to gas chromatography by measuring the recovery of [^{3}H]cortisol.

Dilution: provides for aliquotization of original urinary samples.

Sensitivity and precision

The sensitivity of the procedure varied according to the GC conditions used. Applying standard conditions sensitivity was approx. 1 ng per injected sample corresponding to about 0.02 mg of steroid per 24 h. The coefficient of variation for multiple determinations ($n = 8$) of the whole spectrum of standard compounds was $12 \pm 3\%$ (range 10–20%).

Table 2. Excretion of steroid metabolites in patients with congenital adrenal hyperplasia

Patients				Excreted steroid metabolites (mg/24 h)												
No.	Age (years)	Sex (m/f)	Previous therapy (yes/no)	An 2	Et 3	DHEA 4	11O-An 7	11O-Et 9	11OH-Et 14	11OH-An 15	PT 20	PT'on 29	(PcT) 30	TH-E 31	aTH-E 35	TH-F 36	aTH-F 37
1	4	f	yes	<0.04	<0.04	<0.05	0.54	<0.03	1.17	0.08	2.88	0.62	0.96	0.07	<0.04	<0.04	<0.04
2	5	f	yes	<0.02	0.80	<0.02	0.62	<0.02	0.72	<0.02	0.87	<0.02	0.83	0.14	<0.03	<0.03	0.04
3	7	f	yes	0.25	<0.06	<0.06	<0.05	<0.04	18.74	<0.04	23.30	20.61	0.87	<0.05	0.15	<0.05	0.07
4	9	f	yes	1.16	0.47	<0.05	1.59	0.40	14.23	0.05	20.30	<0.08	2.87	3.13*	<0.07	<0.05	0.39
5	10	f	yes	3.15	0.60	<0.06	<0.07	<0.04	20.70	<0.05	16.90	6.94	0.56	0.80	<0.07	<0.08	0.13
6	12	f	yes	<0.07	<0.07	<0.07	1.26	<0.09	12.21	<0.05	14.90	0.52	2.35	0.57	<0.10	<0.10	<0.10
7	12	f	yes	0.87	<0.06	<0.06	1.76	<0.05	?	<0.04	18.82	0.23	2.73	<0.06	<0.06	0.28	0.11
8	13	f	yes	3.86	1.77	<0.07	<0.09	<0.05	12.30	0.20	16.00	2.74	5.45	2.43*	0.54	0.60	1.50
9	13	f	yes	15.70	4.80	3.75	0.65	<0.10	40.80	0.10	41.30	5.85	2.15	2.40*	<0.10	<0.10	0.40
10	15	f	yes	6.51	2.06	<0.10	<0.14	<0.08	24.90	1.51	37.00	9.81	1.07	2.24*	<0.12	<0.10	<0.11
11	16	f	yes	7.81	6.44	0.32	0.59	0.06	65.30	2.21	122.10	50.60	0.96	4.74*	<0.08	<0.08	0.79
12	21	f	yes	0.31	<0.02	<0.02	0.13	<0.02	2.15	<0.01	3.89	6.77	0.43	0.32	<0.02	<0.02	0.05
13	24	f	no	18.00	24.40	3.21	<0.25	<0.17	61.60	3.48	102.80	7.86	2.90	17.50*	<0.22	13.50	14.60
14	50	f	no	3.37	0.07	0.06	<0.30	0.24	8.64	0.03	5.60	0.85	1.36	0.33	<0.02	<0.02	1.22
15	4	m	no	1.51	1.04	<0.10	0.38	0.07	11.51	<0.08	22.80	2.95	1.00	0.64	<0.10	<0.10	0.31
16	5	m	yes	1.97	0.40	<0.05	0.44	0.05	11.30	<0.05	17.10	3.00	1.82	0.96	<0.05	<0.05	0.01
17	5	m	yes	0.11	<0.02	<0.02	0.28	<0.02	1.38	0.09	3.49*	0.89*	0.93	0.33	<0.03	<0.03	<0.03
18	5	m	yes	0.11	<0.02	0.02	<0.03	<0.02	0.66	<0.02	1.39*	0.20*	1.78	0.02	<0.04	<0.04	<0.04
19	7	m	yes	0.33	0.05	0.37	<0.05	<0.03	0.04	0.11	0.19*	0.03	2.21	0.03	<0.04	<0.03	<0.03
20	7	m	yes	<0.02	<0.02	0.04	<0.04	<0.02	<0.02	<0.02	0.95*	<0.02	1.45	<0.02	<0.02	<0.02	<0.02
21	8	m	yes	0.16	<0.03	<0.03	0.71	<0.02	0.32	0.02	4.84	0.23	1.28	0.40	<0.03	<0.03	<0.03
22	9	m	yes	0.04	<0.03	0.04	<0.04	<0.02	0.11	<0.02	0.32*	0.06	1.50	<0.04	<0.04	<0.04	<0.04
23	15	m	yes	<0.15	<0.15	<0.20	1.14	0.62	5.50	<0.15	31.10	<0.20	1.96	<0.20	<0.20	<0.20	<0.20
Healthy adult men ($n = 5$)				2.86	1.92	0.09		0.04	0.80	0.12	1.19		0.24	1.07		1.04	0.50
range = mean ± 1 SD				−3.48	−3.42	−0.43	−0.06	−0.14	−1.60	−0.42	−1.87	−0.10	−0.36	−2.27		1.24	−0.70
Healthy adult women ($n = 5$)				1.04	1.41			0.11	0.50	0.09	0.54		0.13	1.45		1.14	0.28
range = mean ± 1 SD				−3.18	−3.39	−0.26	−0.30	−0.21	−1.02	−0.31	−1.78	−0.10	−0.31	−2.17		1.66	−0.88

*Indicates positive, indicates negative identification by mass-spectrometry

Statistics

Data are presented as mean ± SD.

RESULTS

Steroid excretion rates of patients with congenital adrenal hyperplasia are given in Table 2. The excretion of pregnanetriol in all but 5 patients was larger than that in healthy, adult controls and the excretion of pregnanetriolone paralleled that of pregnanetriol in 20 patients. The excretion of the glucocorticoid metabolites, tetrahydro-cortisone, allo-tetrahydro-cortisone, tetrahydro-cortisol and allo-tetrahydro-cortisol was predominantly below that of healthy adult controls. Specifically, the excretion of tetrahydro-cortisone was subnormal in 17 patients including the 5 individuals who failed to exhibit an abundance of pregnanetriol excretion. Six female patients (Nos 4, 8, 9, 10, 11 and 13) excreted supranormal amounts of tetrahydro-cortisone. The presence of this steroid in the patients' urine was confirmed by GC-MS (SIM) in each case and homogeneity of the respective peak was demonstrated in one sample (patient No. 13) by recording total ion current.

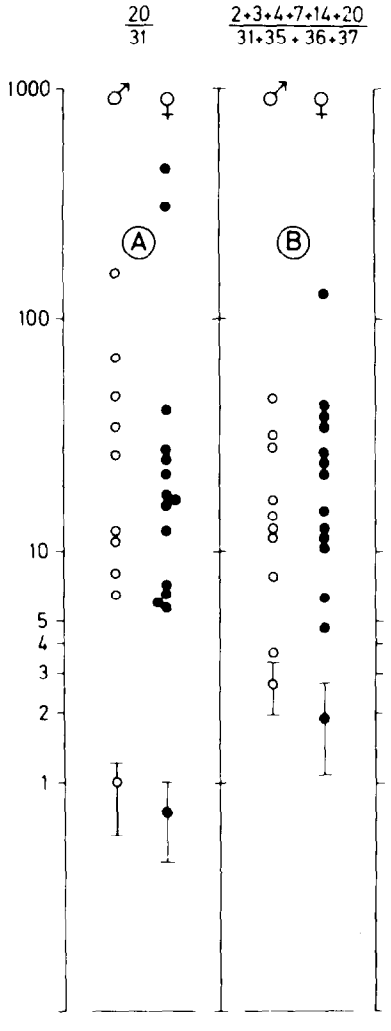

Fig. 1. Excretion of steroids in patients with congenital adrenal hyperplasia and in healthy controls (5 men, 5 women, $\bar{x} \pm$ SD). *A*: ratio of excreted pregnanetriol/tetrahydrocortisone; *B*: ratio of combined excretion rates of androsterone, etiocholanolone, dehydroepiandrosterone, 11-keto-androsterone, 11-hydroxy-androsterone, and pregnanetriol versus the combined excretion rates of tetrahydrocortisone, allo-tetrahydrocortisone, tetrahydrocortisol, and allo-tetrahydrocortisol.

However patients with abundant excretion of tetrahydro-cortisone also presented extensively elevated excretion rates of pregnanetriol. Thus the calculated ratio of excreted pregnanetriol/tetrahydrocortisone ranged from 5.9 to 466 in patients with CAH and was well above that of healthy men (1.0 ± 0.4) and of healthy women (0.7 ± 0.3; Fig. 1A). A similar difference was found in the ratio of the combined excretion rates of androsterone, etiocholanolone, dehydroepiandrosterone, 11-keto-androsterone, 11-hydroxy-androsterone and pregnanetriol versus the combined excretion rates of tetrahydro-cortisone, allo-tetrahydro-cortisone, tetrahydro-cortisol and allo-tetrahydro-cortisol (range in patients with CAH: 3.6–132.7, as compared to 2.7 ± 0.7 in healthy men and to 1.9 ± 0.8 in healthy women (Fig. 1B).

Initial gas chromatographic analysis suggested an increased excretion of pregnenetriol in each single patient, which seemed to exceed that of pregnanetriol in 4 cases. However, additional mass spectrometric analysis of the 4 urine samples following prepurification by thin layer chromatography failed to confirm the presence of pregnenetriol in the patient's urine, whereas pregnanetriol and pregnanetriolone were positively identified.

The excreted amounts of 11-keto-etiocholanolone and 11-hydroxyetiocholanolone in patients with CAH were frequently below the sensitivity of the method and also below that of healthy adults in 19 and 17 patients, respectively. The excretion of the 5α-hydrogenated homologues, 11-keto-androsterone and 11-hydroxy-androsterone however, exceeded that of healthy adults in the urine of 12 and 15 patients, respectively. The predominance of 5α-hydrogenated compounds in patients with CAH is demonstrated by the calculated ratio of excreted 11-keto-androsterone and 11-hydroxy-androsterone versus 11-keto-etiocholanolone and 11-hydroxy-etiocholanolone (Fig. 2).

Excretion rates of etiocholanolone and androsterone exceeded those of healthy adults respectively in 6 and 3 patients with CAH, whereas in the remaining patients the excretion rates of both metabolites was in or below the range of adult controls.

DISCUSSION

In congenital adrenal hyperplasia the compensatory increase in ACTH secretion secondary to

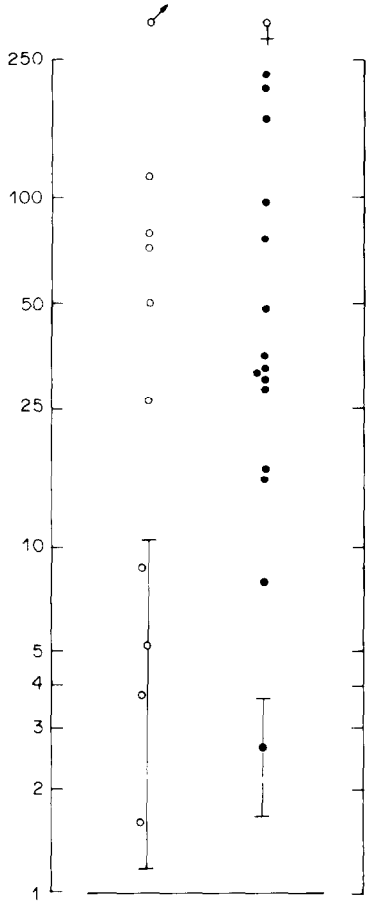

Fig. 2. Excretion of steroids in patients with congenital adrenal hyperplasia and in healthy controls (5 men, 5 women, $\bar{x} \pm SD$). Calculated ratio of excreted 11-ketoandrosterone and 11-hydroxyandrosterone versus 11-ketoetiocholanolone and 11-hydroxyetiocholanolone.

decreased cortisol synthesis stimulates the synthesis of steroids unimpeded by the enzyme deficiency. For this reason patients with 21-hydroxylase deficiency characteristically accumulate 17-hydroxyprogesterone and 21-desoxycortisol and excrete excessive amounts of the respective metabolites, pregnanetriol and pregnanetriolone [7, 8]. Whereas 18 out of 23 patients examined for the purpose of this study presented abundance of excreted pregnanetriol, the excretion of this steroid was in or below the range determined in healthy adults in the remaining 5 patients. This finding may either be due to the short (3 days) glucocorticoid withdrawal prior to the study or to lack of the patients' compliance to discontinue their usual medication on an outpatient basis. However in all 23 patients including the 5 with normal pregnanetriol excretion rates the ratio of excreted pregnanetriol/tetrahydro-cortisone was well above that seen in healthy subjects. The use of tetrahydro-cortisone for this calculation is convenient, since this metabolite is easily quantified in the used gas chromatographic system. Thus the simultaneous determination of pregnanetriol and tetrahydro-cortisone will demonstrate 21-hydroxylase deficiency even in the state of a relatively suppressed pituitary-adrenal axis. A defect in 21-hydroxylase activity was confirmed when the combined excretion rates of several steroids unimpaired by 21-hydroxylase deficiency (androsterone, etiocholanolone, dehydroepiandrosterone, 11-keto-androsterone, 11-hydroxy-androsterone, and pregnanetriol) were compared with the combined excretion rates of 4 glucocorticoid metabolites (tetrahydro-cortisone, allo-tetrahydro-cortisone, tetrahydro-cortisol, and allo-tetrahydro-cortisol).

The expected deficiency in glucocorticoid excretion was seen in most of our patients with CAH, but normal or even supranormal quantities of glucocorticoid metabolites were excreted by a subgroup of 7 female patients. Specifically the excretion of tetrahydrocortisone was above normal in 6 patients. The identity of this metabolite was confirmed in the respective urine samples by additional mass spectrometry using selected ion monitoring and the homogeneity of the gas chromatographic peak was confirmed by recording the total ion current of one sample with a specially high content of tetrahydrocortisone (Patient No. 13).

Whether a defect in the feedback control of ACTH secretion in these patients induced a rise in ACTH secretion even in the presence of increased glucocorticoid concentrations remains speculation since plasma samples for the determination of plasma concentrations of cortisol and ACTH were not obtained. It has to be emphasized, however, that the ratio pregnanetriol/tetrahydrocortisone was well above normal in all of these 7 patients.

An elevated excretion of pregnenetriol is regarded as a characteristic of 3β-hydroxysteroid-dehydrogenase deficiency. In 21-hydroxylase deficiency the excretion of pregnanetriol is supposed to exceed that of pregnenetriol [9, 10]. Following these considerations the apparent preponderance of gas chromatographically determined pregnenetriol over pregnanetriol in 4 of our patients with CAH seemed to indicate an impaired 3β-hydroxysteroid-dehydrogenase. This was unlikely since the patients in question were males without evidence of ambiguous genitalia and since no excess in the excretion of DHEA was observed. Moreover, the CAH-affected brother (patient No. 17) of one of the patients in question (patient No. 20) failed to exhibit a preponderance of pregnenetriol over pregnanetriol excretion. The presence of pregnanetriolone was confirmed by mass spectrometry in one urine sample and this steroid is characteristically absent in 3β-hydroxysteroid-dehydrogenase deficiency [9]. Finally mass-spectrometry confirmed the presence of pregnanetriol, but not of pregnenetriol in all of the urine samples in question suggesting interference of an as yet unidentified steroid metabolite with the initial gas chromatographic determination of pregnenetriol and emphasizing that positive identification

of single steroid metabolites has to be attempted when gas chromatographic analysis reveals unexpected findings. Thus, any results obtained with gas chromatography without this auxiliary method [9, 10] must be regarded as preliminary, even though they are potentially correct.

The relative preponderance of the $C_{19}O_3$ 5α-hydrogenated metabolites, 11-ketoandrosterone and 11-hydroxyandrosterone over their respective 5β-hydrogenated homologues, 11-ketoetiocholanolone and 11-hydroxyetiocholanolone, may be explained by the patients' relative lack of glucocorticoids, since the former two metabolites are predominantly formed from 21-desoxycortisol and from 11-keto-(hydroxy) androstenedione, whereas the latter are predominantly metabolites of cortisol and cortisone [11]. It has been shown *in vitro* that 5α-reductase is inhibited by ACTH and by testosterone [12]. Since in congenital adrenal hyperplasia concentrations of both ACTH and of testosterone may be expected to be above rather than below normal, it is unlikely that the influence of either hormone accounts for the predominance of 5α-reduced $C_{19}O_3$-metabolites observed in our patients.

In man, etiocholanolone and androsterone are the main metabolites of dehydroepiandrosterone and testosterone [13]. Since impaired 21-hydroxylase activity is associated with a rise in the production of dehydroepiandrosterone and testosterone, both metabolites are usually excessively produced by patients with 21-hydroxylase deficiency [14]. Several of our patients who were older than 10 years presented elevated excretion rates of androsterone and etiocholanolone. It is difficult to comment on the low excretion rates in the younger patients, since data on age-matched controls are not available. With regard to the delayed rise in adrenal androgens as compared to glucocorticoids following withdrawal of exogenous glucocorticoids [15] the short period of glucocorticoid withdrawal in our patients may help to explain these observations. An increase in the relative share of gonadal steroid production to overall steroid excretion and/or changes in the activity of 17,20-lyase [16] may also contribute to the age-related differences in the excretion of androsterone and etiocholanolone.

In conclusion, we feel that the value of capillary gas chromatography in the characterization of adrenal enzyme defects is the possibility to determine simultaneously high levels of precursors and low levels of products of the affected enzymatic step even in the state of a partly-suppressed pituitary-adrenal axis, since neither the isolated demonstration of normal excretion rates of precursors nor of elevated excretion rates of the respective product excludes the diagnosis in a specific patient. The method is limited by the presence of unidentified steroid metabolites. Thus, positive identification by mass spectrometry has to supplement gas chromatographic analysis where necessary in order to avoid erroneous interpretation of the obtained data.

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REFERENCES

1. Luyten J. A. and Rutten G. A. F. M.: Analysis of steroids by high-resolution gas-liquid chromatography. II. Application to urinary samples. *J. Chromat.* **91** (1974) 393–406.
2. Pfaffenberger C. D. and Horning E. C.: High-resolution biomedical gas chromatography. Determination of human urinary steroid metabolites using glass open tubular capillary columns. *J. Chromat.* **112** (1975) 581–594.
3. Shackleton C. H. L. and Honour J. W.: Simultaneous estimation of urinary steroids by semiautomated gas chromatography. Investigation of neonatal infants and children with abnormal steroidogenesis. *Clin. chim. Acta* **69** (1976) 267–283.
4. Völlmin J. A.: High resolution gas chromatography of urinary steroids on glass capillary columns. *Clin. chim. Acta* **34** (1971) 207–214.
5. Viinikka L., Jänne O., Perheentupa J. and Vihko R.: Congenital adrenal hyperplasia. Plasma and urinary steroid conjugates in seven children with steroid 21-hydroxylase deficiency. *Clin. chim. Acta* **48** (1973) 359–365.
6. Chambaz E. H. and Horning E. C.: Conversion of steroids to trimethylsilyl derivatives for gas phase analytical studies: reaction of silylating agents. *Analyt. Biochem.* **30** (1969) 7–24.
7. Butler G. C. and Marrian G. F.: Isolation of pregnane-3,17,20-triol from urine of women showing the adrenogenital syndrome. *J. biol. Chem.* **119** (1937) 565–572.
8. Cox R. I. and Finkelstein M.: Pregnane-3α,17,20-triol and pregnane-3α,17,20α-triol-11-one excretion by patients with adrenocortical dysfunction. *J. clin. Invest.* **36** (1957) 1726–1735.
9. Bongiovanni A. M., Eberlein W. R. and Moshang T. Jr: Urinary excretion of pregnanetriol and Δ5-pregnenetriol in two forms of congenital adrenal hyperplasia. *J. clin. Invest.* **50** (1971) 2751–2754.
10. Bongiovanni A. M.: Urinary steroid pattern of infants with congenital adrenal hyperplasia due to 3-beta-hydroxysteroid dehydrogenase deficiency. *J. steroid Biochem.* **13** (1980) 809–811.
11. Keutmann E. H. and Mason W. B.: Individual urinary 17-ketosteroids of healthy persons determined by gas chromatography: biomedical and clinical considerations. *J. clin. Endocr. Metab.* **27** (1967) 406–420.
12. Kitay J. I., Coyne M. D. and Swygert N. H.: Effects of hypophysectomy and administration of cortisone or ACTH on adrenal 5α-reductase activity and steroid production. *Endocrinology* **89** (1971) 432–438.
13. Fukushima D. K., Bradlow H. L., Dobriner K. and Gallagher T. F.: The fate of testosterone infused intravenously in man. *J. biol. Chem.* **206** (1954) 863–874.
14. Bradlow H. L. and Gallagher T. F.: Metabolism of 11β-hydroxyΔ4-androstene-3,17-dione in congenital adrenal hyperplasia. *J. clin. Endocr. Metab.* **19** (1959) 1575–1580.
15. Cutler G. B., Davis S. E., Johnsonbaugh R. E. and Loriaux D. L.: Dissociation of cortisol and adrenal androgen secretion in patients with secondary adrenal insufficiency. *J. clin. Endocr. Metab.* **49** (1979) 604–609.
16. Schiebinger R. J., Albertson B. D., Cassorla F. G., Bowyer D. W., Geelhoed G. G., Cutler G. B. Jr and Loriaux D. L.: The developmental changes in plasma adrenal androgens during infancy and adrenarche are associated with changing activities of adrenal microsomal 17-hydroxylase and 17,20-desmolase. *J. clin. Invest.* **67** (1981) 1177–1182.