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GAS–LIQUID CHROMATOGRAPHY IN THE DIAGNOSIS OF CONGENITAL ADRENAL HYPERPLASIA*

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SUMMARY

A gas–liquid chromatographic method to measure 17-hydroxyprogesterone, pregnanetriol, and 17-hydroxypregnanolone in serum is described. This method has been applied to the diagnosis of congenital adrenal hyperplasia with success. All three compounds are elevated and fall rapidly upon treatment. This method, as described, does not detect these steroids in normal human serum, although the levels reported by some radioimmunoassay techniques are high enough to have been discerned.

INTRODUCTION

In an attempt to simplify the laboratory diagnosis of congenital adrenal hyperplasia due to deficiency of 21-hydroxylase, a method was devised employing gas–liquid chromatography (g.l.c.) to measure the 17-hydroxyprogesterone and related steroids in a small volume of serum. The specific steroids studied and the trivial names and abbreviations employed herein are as follows: 5β-pregnane-3α,17α, 20α-triol (pregnanetriol, PT); 3α,17α-dihydroxy-5β-pregnan-20-one (17-hydroxy-pregnanolone, HPne); 17α-hydroxypregn-4-ene-3,20-dione (17-hydroxyprogesterone, 17HP). It is often difficult to obtain accurate twenty-four hour urine specimens in the very young and furthermore such collection is time consuming. Although the sensitivity of this method is not so great as with radioimmunoassay, it has been possible to recognize the disorder within a few hours.

MATERIALS AND METHODS

Two ml of serum were generally employed, although in the presence of elevated levels of the critical steroids as little as 1·0 ml was satisfactory. This was extracted three times with five vol. of ethyl ether. The ether was washed twice with one third vol. of 0·1 N sodium hydroxide and twice with water. Following evaporation the residue was partitioned between 2·0 ml ethanol and 3·0 ml low boiling petroleum ether. This step was necessary to remove cholesterol and other unidentified impurities which were found to obscure the critical peaks of the gas–liquid chromatograph.

After the prior extraction, the remainder of the serum was diluted to 10·0 ml with 0·1 M sodium acetate buffer pH 4·5 and incubated overnight at 45° with 10,000 units mammalian liver β-glucuronidase. This was extracted three times with 20 ml methylene chloride, washed and partitioned as above. This extract of the conjugated steroids was further purified on a column of silica gel (2% water, 1 × 6 cm). The residue was applied in methylene chloride, washed with 10 ml 1% ethanol in methylene chloride and the eluate obtained with 15 ml of 12% ethanol in methylene chloride was employed for application to g.l.c. Correction for recovery was achieved by adding 1·0 μg % cholestane to the serum prior to the first extraction for the free steroids which contained 17-HP and 1·0 μg % prednisolone prior to glucuronidase hydrolysis of the remainder.

Gas–liquid chromatography was carried out in a Barber–Coleman instrument, model 5000 with a hydrogen flame ionization detector and chart recorder. Glass columns of 6 ft length and 3 mm i.d. packed with 100–120 mesh Gas–Chrom Q (Applied Science Labs, Inc., State College, Pa.) coated with QF-1 or OV-1 were employed.

The identification and quantitation of the steroids by g.l.c. was conducted in three steps. First, 1/5 aliquot of each extract representing "free" steroid was injected onto OV-1 at 232°C, 22 psi nitrogen wherein the relative retention time (RRT) to etiocholanolone was required to correspond to standards run the same day.

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Table 1. Relative retention times of steroid derivatives to etiolcholanolone

Steroids	Derivatives and column employed: FREE OV-1	BSA QF-1	MOX-TBT QF-1
17-Hydroxyprogesterone	3·030	0·831	0·902
17-Hydroxypregnanolone	2·20	1·180	0·380
Pregnanetriol	2·70	0·934	0·502

Table 2. Recovery of 17-hydroxyprogesterone added to stripped pool of adult male serum. (All figures as μg per cent)

Specimen	Amount steroid added	Recoveries in duplicate specimens	
1	0	0	0
2	0·200	0	0
3	0·350	0·100	0·100
4	0·500	0·400	0·420
5	0·750	0·710	0·800
6	1·00	1·00	1·05
7	20·00	18·10	20·95
8	50·00	50·10	52·10

Second, the N,-bis-(trimethylsilyl)-acetamides (BSA) were prepared according to Chambaz and Horning [2] with the remainder and injected onto 3% QF-1, 208°C, 12 psi nitrogen. This BSA derivative was also required to conform to standards, although in the case of 17-HP, which does not react with this reagent, it was the free steroid on an alternate column phase that was observed. Finally, the remainder (now 3/5) was treated with 2% methoxyamine HCl in pyridine and a mixture of trimethylsilyl-imidazole/N,O-bis-trimethylsilyl-acetamide/trimethylchlorosilane (3/3/2), designated MOX-TBT [2], and injected onto QF-1. This last derivative was used for quantitation by planimetry since it yielded the sharpest and strongest peaks. In the results reported the RRT corresponded in all three instances to the compounds reported. The RRT of these various forms are shown in Table 1. In our experience whenever the MOX-TBT derivatives gave distinct sharp signals, the other two forms of the steroid were always detectable so that the MOX-TBT alone probably suffices for routine determinations. We have not observed interference with this derivative from contaminants.

For studies of recovery of added steroids, pooled adult plasma which had been stripped with activated

Table 3. Steroid levels in serum (μg per cent)

Condition: adrenal hyperplasia	Sex	Age	17HP	PT	HPne
1.	F	1 week	23·0	22·0	51·2
2. Two specimens	F	2 weeks	53·7	151·0	151·0
			64·5	177·5	170·5
24 h cortisol treatment			20·0	11·0	26·0
48 h treatment			0	0	0
3.	F	6 weeks	38·6	98·7	28·1
4.	F	6 weeks	32·4	119·0	100·0
5.	F	8 weeks	41·6	137·0	19·0
6. Intravenous cortisol					
1 h			0	93·0	14·0
2 h			0	60·0	9·5
4 h			0	46·5	6·0
12 h			0	0	0
7.	M	1 yr	31·6	236·0	55·0
8.	M	2 yr	20·8	140·5	172·5
9.	M	4 yr	23·0	180·0	46·0
10.	M	21 yr	60·8	140·5	172·0
Normals					
1. Ten separate pools adult male plasma	M	18–46 yr	0	0	0
2. Six cord bloods	M–F		0	12·0 ± 8·2	0
3. Children [22]	M–F	4–16 yr	0	0	0
Miscellaneous					
1. Hypopituitarism [8]	M–F	6–12 yr	0	0	0
2. Adrenal adenoma [1]	F	5 yr	0	19·0	0

charcoal was employed. To each 100 ml plasma was added 1·0 g Norit A; this was shaken 2 min and filtered through Whatman No. 1 paper.

RESULTS

In normal male adult serum we were unable to detect 17-hydroxyprogesterone although aliquots as large as 10 ml of serum were applied to g.l.c. In stripped plasma from normal adult males it was not possible to detect 17-hydroxyprogesterone in quantities less than 0·200 μg %, equivalent to 0·040 μg in 2 ml of serum, the largest quantity applied to g.l.c. in these experiments. Between 0·200–50·0 μg % of added 17-hydroxyprogesterone agreement between duplicate specimens was good (Table 2) and recovery was 80–102% at levels of 0·50 μg % or greater. In these experiments 2·0 ml of serum was used, as described above.

In six normal newborn infants (three male and three female) 17-hydroxyprogesterone was not detected in their cord blood but 0–20 μg % of pregnanetriol was found. In none of the normal children, adults, or single case of virilizing adrenal tumor was 17-hydroxyprogesterone or HPne found. Pregnanetriol was detected in the serum from the adrenal tumor. The results are shown in Table 3.

In all cases of congenital adrenal hyperplasia there were high levels of 17-hydroxyprogesterone, pregnanetriol and 17-hydroxypregnanolone. Usually the pregnanetriol was highest of the three. The 17-hydroxyprogesterone dropped rapidly whereas pregnanetriol and 17-hydroxypregnanolone tended to fall more slowly upon treatment with cortisol. Although the determination of the reduced derivatives required more than one day, the measurement of 17-hydroxyprogesterone was completed within a few hours and served to establish the diagnosis. Furthermore, all cases of congenital adrenal hyperplasia included herein were confirmed by classical urinary steroidal studies described previously [1].

DISCUSSION

In laboratories where gas chromatography is a routine procedure it is more practicable to employ this method for the diagnosis of congenital adrenal hyperplasia. It is not feasible, except in some special situations, to immediately conduct the radioimmunoassay for steroids in order to make this diagnosis in a single specimen. These results indicate that gas chromatography may establish the diagnosis within a few hours. Although it is true that the pregnanetriol may be the highest of the diagnostic steroids, and requires at least overnight hydrolysis, the 17-hydroxyprogesterone alone appears to be reliable and may be quickly determined.

It must be conceded that this method is not sufficiently sensitive as the radioimmunoassay or competitive protein binding and thus cannot detect these compounds in normal serum. Strott *et al.* [3] report levels of 17HP to be 0·094 μg per cent in normal men with competitive binding, a quantity which our method would not detect. Nor would it be feasible to suggest the employment of large volumes of serum for analysis in order to establish the diagnosis of adrenal hyperplasia merely to contrive a technique which measures the "normal" quantities. Similarly, Stewart-Bentley and Horton [4] and Luisi *et al.* [5] employing competitive protein binding have been able to accurately measure 17-hydroxyprogesterone in the plasma of normal men and women reporting levels well below 0·200 μg %. These methods require preliminary chromatographic purification which is time consuming, although admittedly they are able to define normal ranges. On the other hand, Abraham *et al.* [6] employing radioimmunoassay and a presumably specific antibody, report levels as high as 2·1 μg % in normal adult males and in females 0·400 to 1·56 μg %, depending on the phase of the cycle. These are levels which we should have expected to occasionally detect in some of our adult pools. This we have been unable to do. The g.l.c. technique is more simple and offers advantages in arriving at the rapid diagnosis of congenital adrenal hyperplasia.

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