A Routine Method for the Analysis of Urinary Steroids by Gas-Liquid-Chromatography

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Many advances have been made recently in methods for analysis of steroids with Gas-Liquid-Chromatography (GLC).[1-14]

However, thechnological limitations have constituted barriers to the effective use of these methods in clinical laboratories. Recent works suggest that these limitations have been largely removed through the development of new phases, new reagents and new procedures. Especially, the Horning's method[13)14)] seems to be the best, because this method was devised for separating C-19 and C-21 human urinary steroids in a single procedure and did not require a purification step specific for the group of compounds being analysed such as other methods and they seemed to give more valuable information to the clinicians than other methods. But this method demands much time for incubation and preparation of material and identification of analysed steroids is more difficult. In order to use this method as a routine clinical laboratory method, we have made several attempts with modifications. We have gotten several interesting sets of data and gained confidence in using this as a routine method. We wish to describe a modified two step hydrolysis technique which uses simultaneous enzymatic hydrolysis and extraction and ethyl acetate extraction followed by usual acid hydrolysis, and the benefit of 2 kinds of internal standards.

METHOD

Principle :

Steroid glucuronides in urine are hydrolysed by β-glucuronidase and simultaneously extracted by ethyl acetate containing two kinds of internal standards with the instrument shown in Fig. 1 and 2. Then the steroid sulfates are hydrolysed by acid hydrolysis technique and extracted by ethyl acetate. Ethyl acetate containing free steroids is washed with a 2-N NaOH and saturated NaCl solution. TMSi and Mo-TMSi derivatives of steroids are prepared by Horning's method using methoxylamine hydrochloride and BSA and analysed by temperature programing Gas-Chromatography.[13-16)]

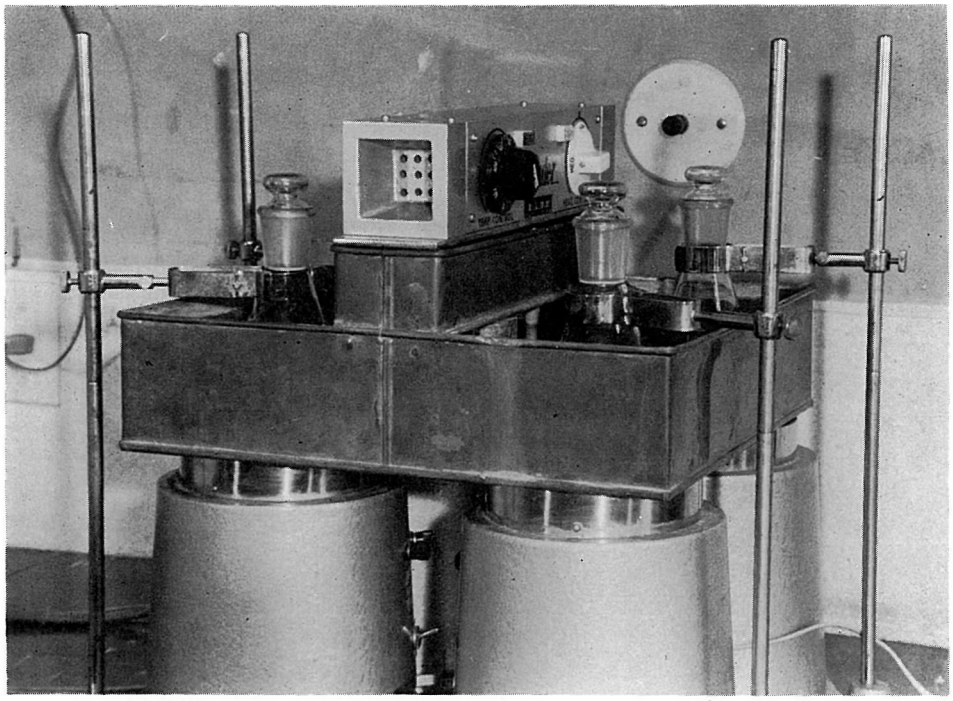

Fig. 1. General view of the arrangement for simultaneous enzymatic hydrolysis and extraction.

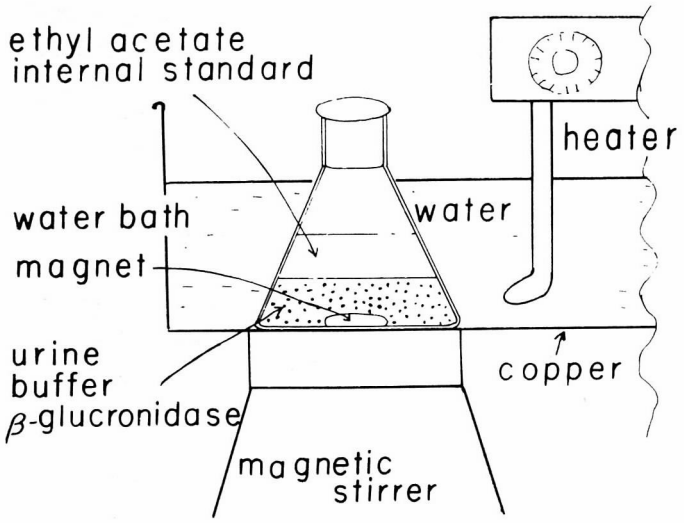

Fig. 2. Illustration of the arrangement (Fig. 1).

Instruments :

1. Shimadzu GC-1C Gas-Chromatography arranged for temperature programming with hydrogen flame ionization detection system, equipped with a dual glass column system of 3 m in length and 0.4 cm OD, 1 % OV-1 column (support, 80–100 mesh Shimalate W).
2. Magnetic stirrers equipped with a water bath for simultaneous enzymatic hydrolysis and extraction (Fig. 1 and 2) made so that the flasks might constantly be held at 48°C and urine and ethyl acetate might be mixed during the hydrolysis.
3. 300 ml flask with a standard joint at the neck.
4. Hamilton microsyringe 10 μl.
5. 60°C water bath.

Reagents and Solvents :

1. β-glucuronidase (Tokyo Zoki, Optimal pH 5.0)
2. Acetate buffer (pH 5.0)
3. 1 N NaOH
4. 1 N HCl
5. 2 N NaOH
6. Saturated NaCl solution
7. Na_2SO_4
8. Pyridine
9. 5 % Na_2CO_3 + 10 % NaCl
10. N, O-Bis (trimethylsilyl) acetamide (BSA) (Tokyo Kasei)
11. Internal standard solution :
    Dissolve n-tetracosane (Gas-Chro-Kogyo) and cholesterol butyl ether (Sigma) in ethyl acetate in concentration of 0.1 mg/ml and store in ampules.
12. O-Methylhydroxylamine hydrochloride (Tokyo Kasei)
13. Ethyl acetate

Procedure :

An aliquot (1/20) of a 24hr urine sample is adjusted to pH 5.0 with 0.1N NaOH or 0.1N HCl, 10 ml acetate buffer followed by addition of 35,000 μ of β-glucuronidase, and transferred to the 300 ml flask. Ethyl acetate, 50 ml, and internal standard solution, 1 ml, are added. The flask is fitted with a magnetic stirrer system. On the magnetic stirrer, the urine phase and the ethyl acetate phase are continuously mixed at the temperature 48°C for more than 8 hours (overnight) so that the enzyme hydrolysis of steroid glucuronides and the simultaneous extranction of free steroids by ethyl acetate might be performed. After the incubation, the flask is allowed to stand for several minutes, the ethyl acetate phase is removed and the urine phase is reextracted with an additional 50 ml of ethyl acetate, and is again separated from the ethyl acetate and after the addition of 10 ml conc HCl the flask containing urine is placed in 100°C water for 15

minutes in order to accomplish acid-hydrolysis. Free steroids are extracted by ethyl acetate upon shaking. Gathered ethyl acetate from enzyme hydrolysis and acid hydrolysis is partitioned 2 or 4 times with the same amount of 2 N-NaOH until no further pigments are removed, and twice by the saturated NaCl solution. The ethyl acetate is dried over anhydrous sodium sulfate under a stream of air in a 60°C water bath. The dried extract is dissolved in 1 ml pyridine containing 0.1 mg O-Methylhydroxylamine hydrochloride and incubated in a 60°C water bath for 3 hours. The pyridine is evaoperated under the nitrogen stream at 60°C. The residue is dissolved again in 30 ml of ethyl acetate, which is washed twice with 10 % Na_2CO_3 + 5 % NaCl. Again, the ethyl acetate is dried over anhydrous sodium sulfate and removed by nitrogen stream at 60°C. The residue is silylated in 0.5 ml BSA for 20 min. at room temperature. 1 μl of BSA solution is introduced into the injection part of the Gas-Chromatography by a 10 μl Hamilton microsyringe. Separation are carried out by starting the isothermal temperature at 210°C for 20 min, followed by temperature programming at a rate of 1 C/min. to 280°C. The flow rates of carrier gas of both columns measured at 210°C are 40 ml/min., which may skillfully be changed independently for the prevention of base line drifting due to imbalance of both column conditions and temperature rise. The injection block ("flask heater") temperature is 210°C and the detector chamber temperature is 300°C. The chart speed is 0.5 cm/min.

Identification and measurement :

TMSi and MO-TMSi derivatives of known pure steroids and the internal stan-

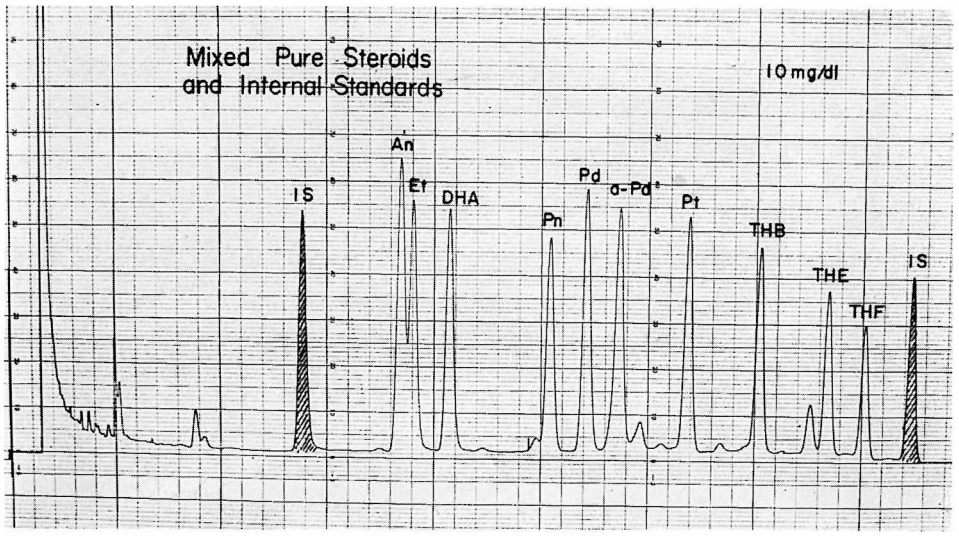

Fig. 3. Chromatogram of mixed pure steroids and internal standards in each concentration of 10 mg/dl: IS=Internal standard (left-n-tetracosane, right-cholesterol butyl ether), An=androsterone, Et=etiocholanolone, DHA=dehydroepiandrosterone, Pn=pregnanolone, Pd=pregnanediol, a-Pd=allopregnanediol, Pt=pregnanetriol, THB=tetrahydrocorticosterone, THE=tetrahydrocortisone, THF=tetrahydrocortisol.

dards mixture are separated by this GLC condition, and the response factors (with respect to the first internal standard n-tetracosane) can be determined for each steroid derivative (Fig. 3). A determination of the amount can be achieved by using the peak area measurement (triangulation), namely the product of peak height and width at half peak height. The peak area on the chromatogram appears to be directly proportional to the amount of steroid. By using both of the internal standards, this pure steroid chromatogram and these response factors, each steroid peak of the sample is able to be identified and measured without difficulties (Fig. 4 and 5).

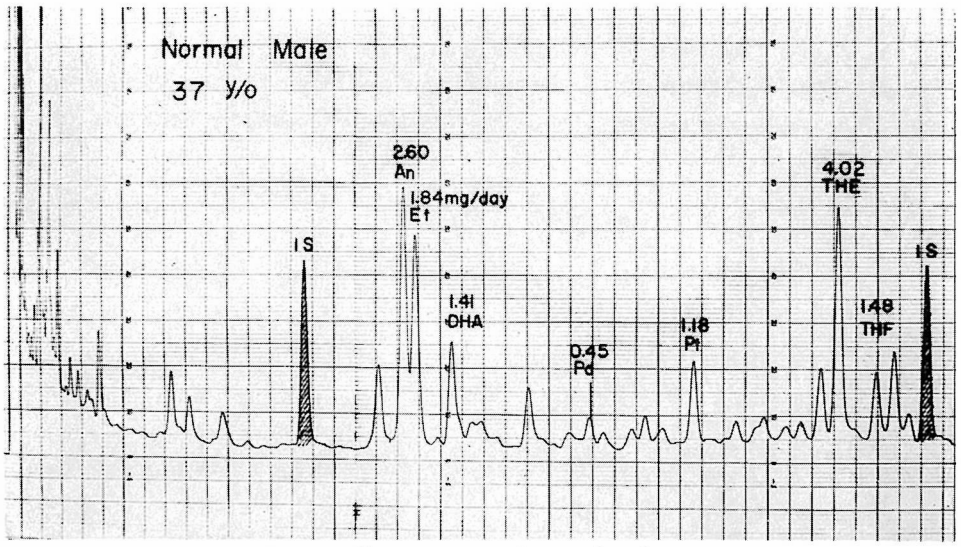

Fig. 4. Chromatogram of normal male.

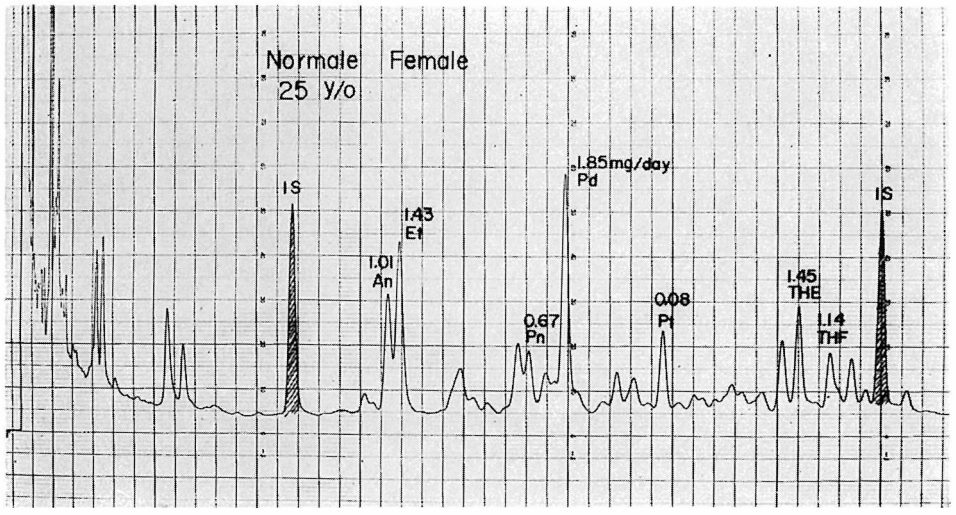

Fig. 5. Chromatogram of normal female.

CASES

Case 1 : These samples are from a 6 year-old male patient with typical signs and symptoms of masculinization. Fig. 6 shows markedly increased androsterone and pregnanetriol, and decreased glucocorticoid metabolites. Administrations of ACTH (Fig. 7), metopirone (Fig. 8), and dexamethasone (Fig. 9) showed interesting responses when compared to each other. During treatment with cortisol the chromatogram showed decreased androsterone, etiocholanolone and pregnanetriol, and the presence of cortisol metabolites such as THE and THF, becoming more similar to the pattern of a normal male.

Case 2 and Case 3 showed similar results to case 1. In Fig. 13 and 14, pregnant females showed increased pregnanediol and other progestogen metabolites. Sheehan's disease and primary hypothyroidism showed markedly decreased urinary steroids (Fig. 15, 16, 17).

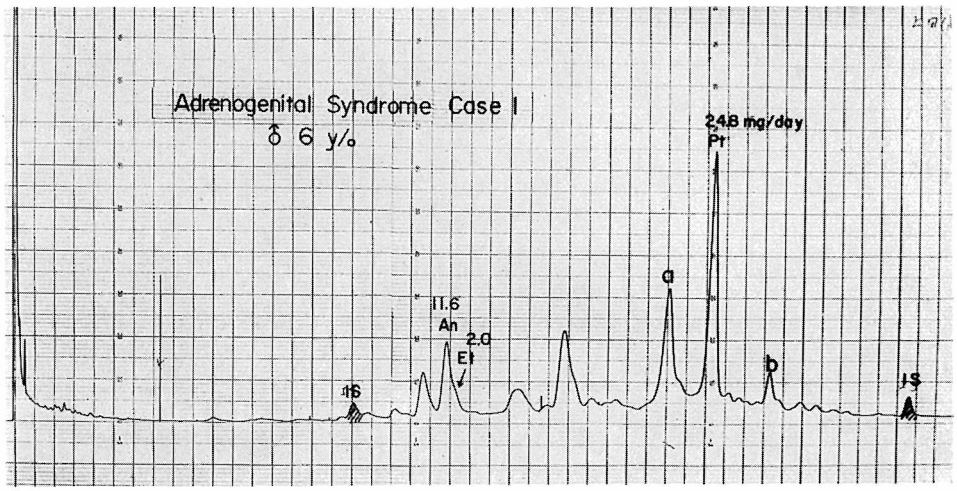

Fig. 6. Chromatogram of adrenogenital syndrome case 1
a = pregnanetetrol ?
b = pregnanetriolone ?

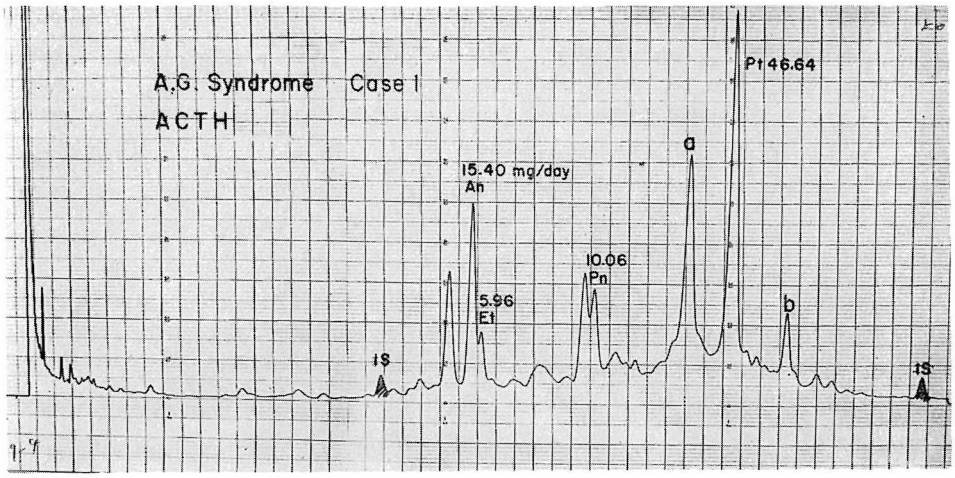

Fig. 7. Chromatogram of adrenogenital syndrome case 1 stimulated by ACTH.

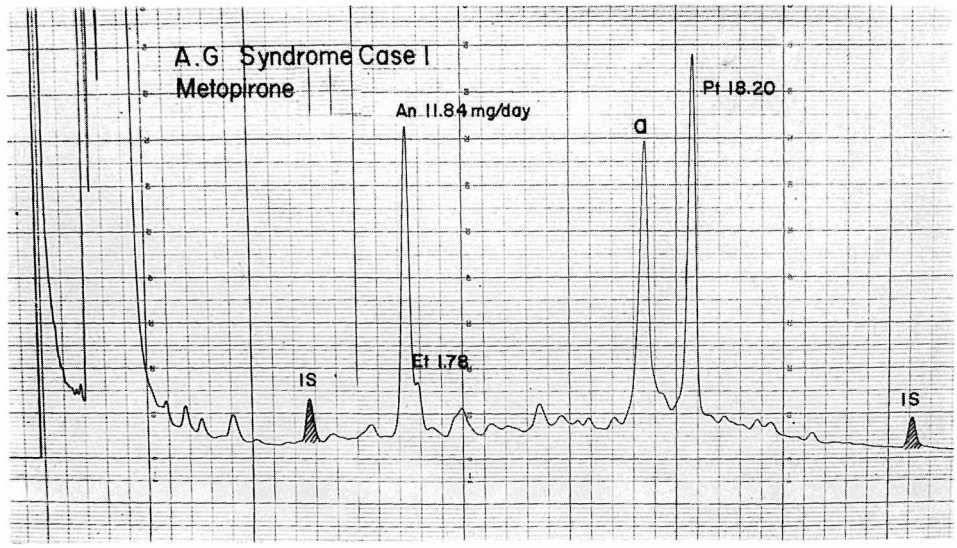

Fig. 8. Chromatogram of adrenogenital syndrome case 1 stimulated by metopirone.

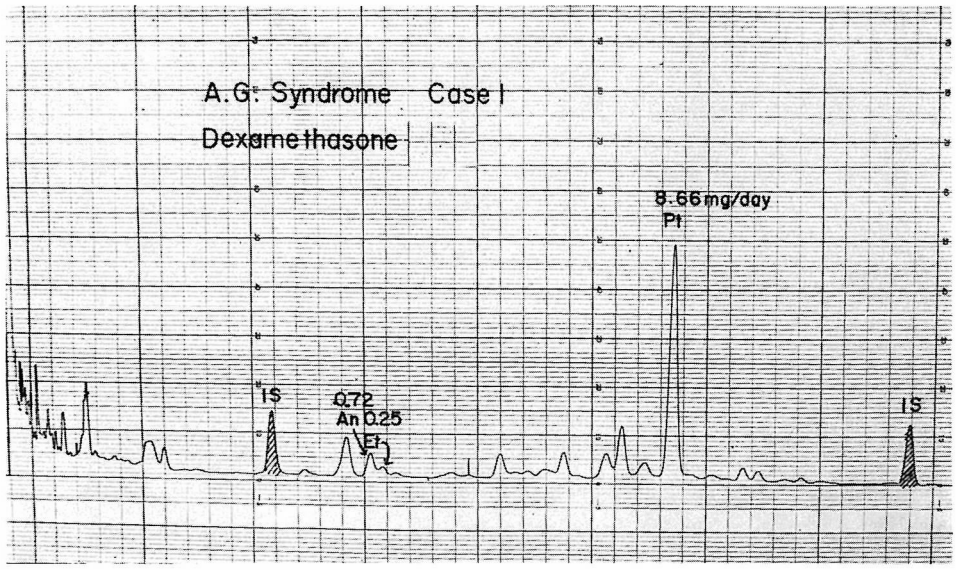

Fig. 9. Chromatogram of adrenogenital syndrome case 1 stimulated by dexamethasone.

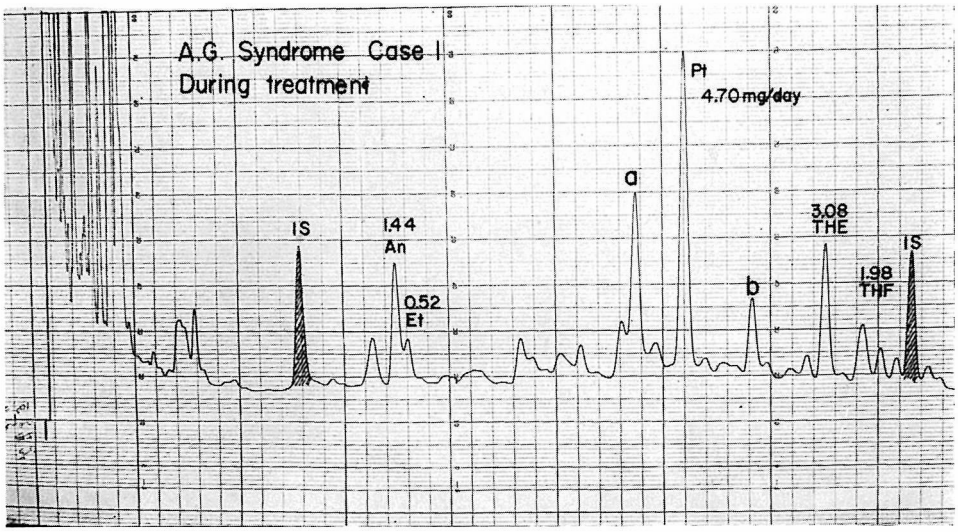

Fig. 10. Chromatogram of adrenogenital syndrome case 1 during treatment.

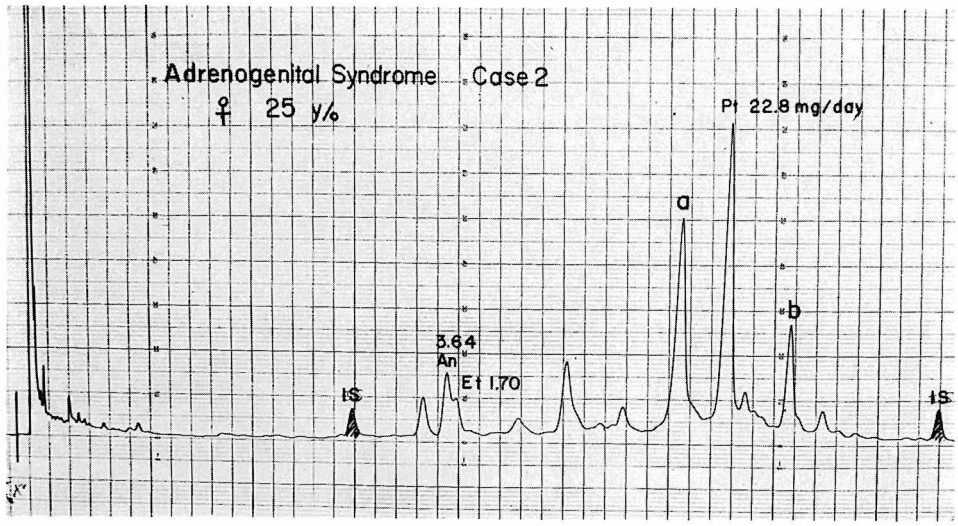

Fig. 11. Chromatogram of adrenogenital syndrome case 2.

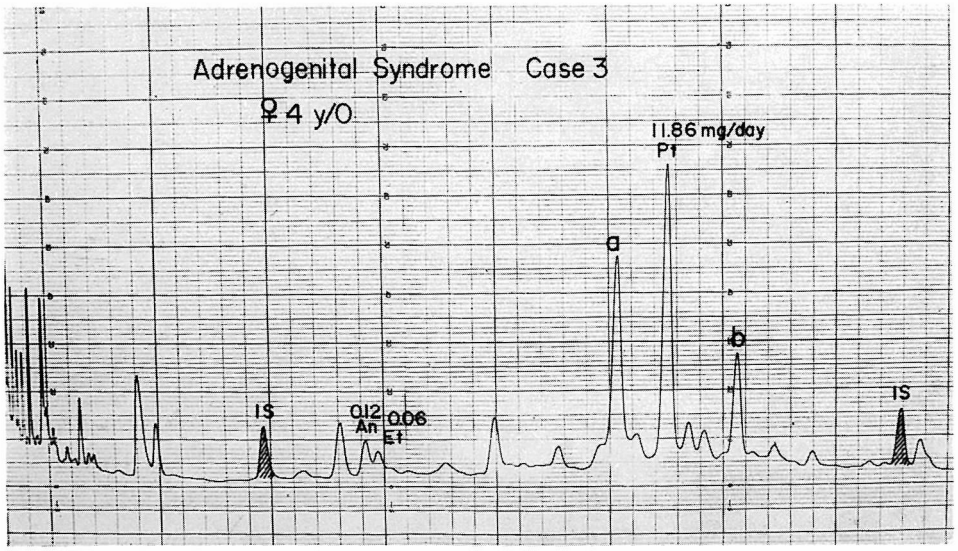

Fig. 12. Chromatogram of adrenogenital syndrome case 3.

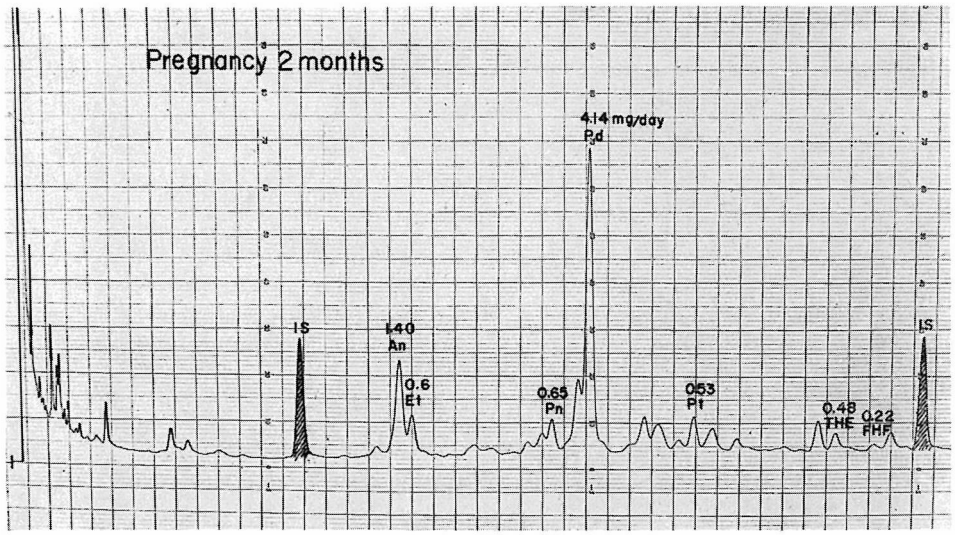

Fig. 13. Chromatogram of a pregnant woman of 2 months.

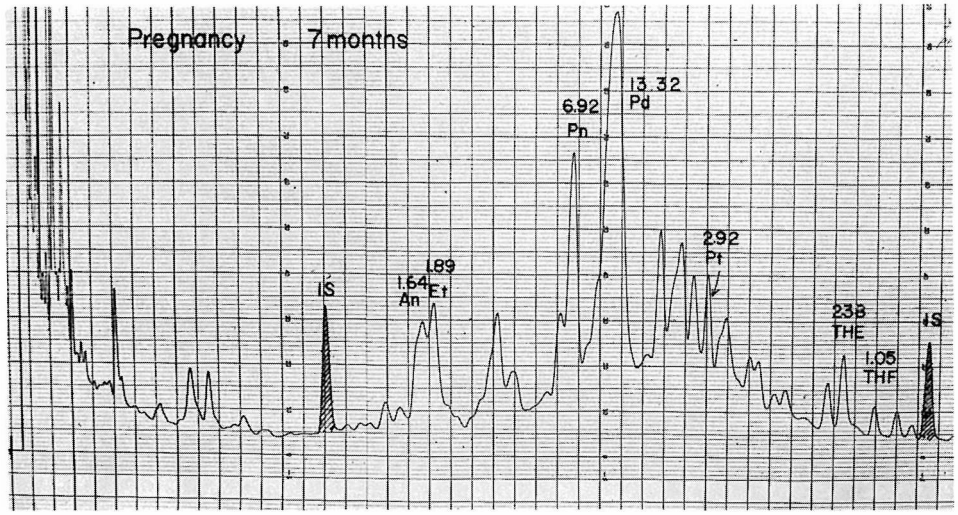

Fig. 14. Chromatogram of a pregnant woman of 7 months.

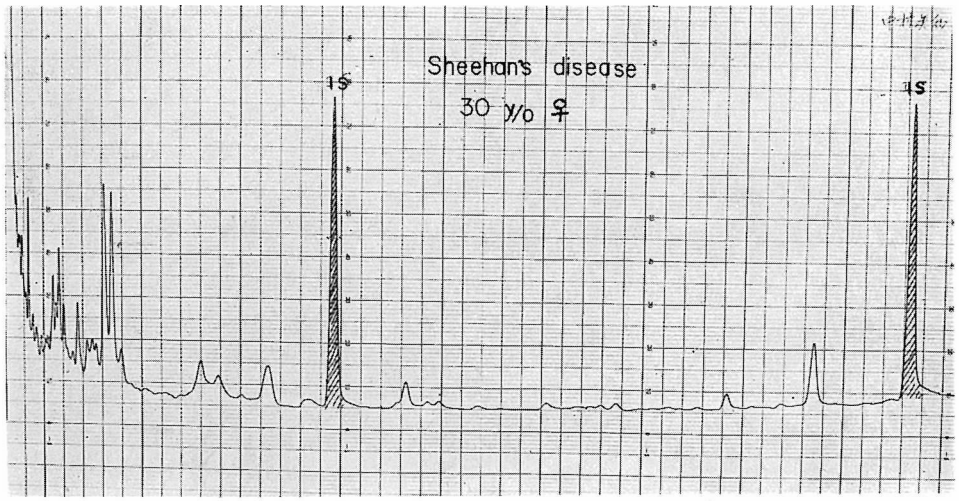

Fig. 15. Chromatogram of Sheehan's disease.

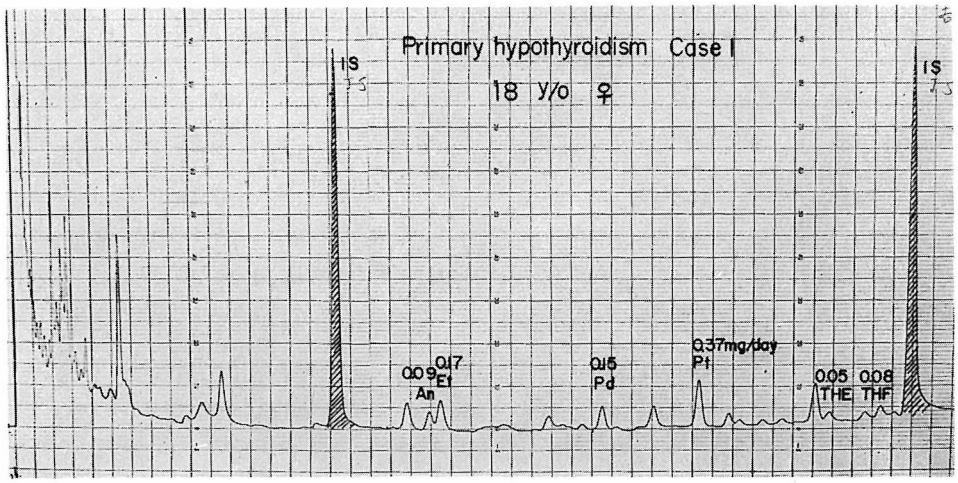

Fig. 16. Chromatogram of primary hypothyroidism case 1.

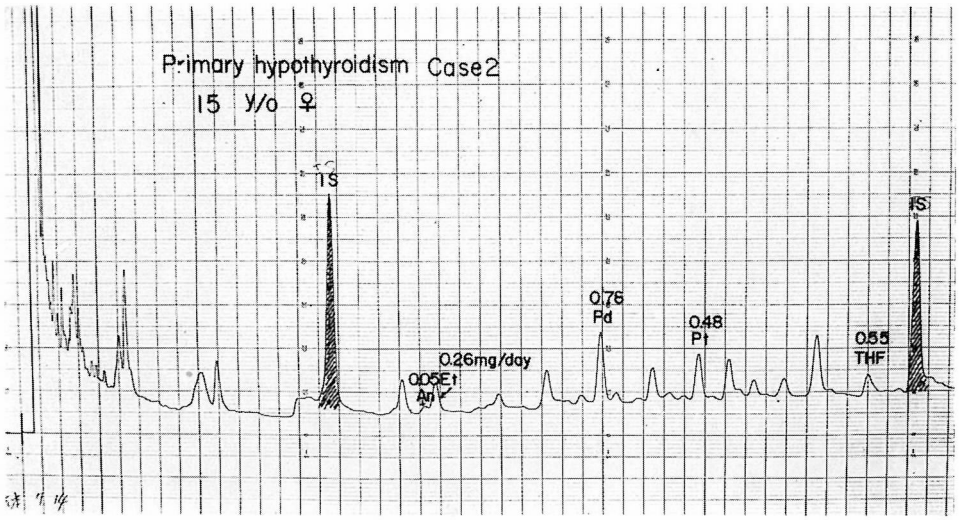

Fig. 17. Chromatogram of primary hypothyroidism case 2.

DISCUSSION

C-21 steroids are so labile that we cannot use acid hydrolysis or solvolysis and we must use enzymatic hydrolysis although the problem of inhibitors in urine remains unsolved. Enzymatic hydrolysis with β-glucuronidase needs a relatively long incubation time[13)14)] which is undesirable in a routine method. With the rapid turnover in hospitals today, the patient may be out of the hospital before the analytical result is obtained if a protracted hydrolysis is used. The enzyme reaction is the same as other chemical reactions with respect to that substrate decrease and enzymatic product accumulation make the speed of enzymatic reactions slow, and on a certain ratio of substrate and product enzyme reaction system reaches to an equilibrium to stop reaction. With this simultaneous enzymatic hydrolysis and extraction method, we have made an attempt to remove the product (free steroids) from the enzyme reaction system continuously and to prevent slowing and stopping of the enzyme reaction and we have been able to shorten the incubation time to 8 hours with the help of an elevated temerature of 48°C. We usually incubate overnight and the next morning finish the incubation and get on with the procedure, so that we can report the data to the clinician in 2 days. In addition in an organic solvent than in an aquaeous solution (especially tetrahydrocortisol), less transformation, artefact formation and destruction were found with this method. In order to identify the separated steroid peak, we cannot use the retention time technique in temperature programming separation. Horning et al.[14)] postulated the MU value which might be a wonderful idea. However, that consumes much

time and needs exactly the same condition of GLC during steroid analysis and during paraffin's analysis. We tried to simplify the identification of steroid peaks by means of double internal standards which sandwich the analysed steroid peaks show in the figures. On comparison with pure steroids mixture analysis, most of the steroid peaks are able to be determined. Internal standards are added at the point of simultaneous enzyme hydrolysis and extraction so that the loss of steroids in the procedure might be minimal on the chromatogram. The chromatogram is very complex and occasionally gives a difficult peak for analysis, so the chromatogram had better used not only for looking at the pattern such as in serum electrophoresis but also for measurement when interpreting the data. We make a copy of the chromatogram to send to the clinic. Typical chromatograms obtained from a normal male and a normal female are illustrated in Figure 4 and 5. A normal male has more urinary steroids than a normal female except for pregnanediol which is changeable according to the menstrual cycle. This sample was collected during the luteal phase. R. Rivera et al.[11] stated that a high urinary pregnanediol level was a good indication that ovulation had taken place. Adrenogenital syndrome (Case 1) showed markedly increased pregnanetriol.[17] We lowered the gas-chromatograph sensitivity so as not to scale out the pregnanetriol peak. However, this resulted in diminution of the internal standard peaks. In order to get more accurate measurement, we were able to use smaller amounts of urine than usual or a greater amount of internal standards. By stimulation of ACTH each peak responded well. The administration of metopirone caused the disappearance of pregnanetriolone which may be a sign of the inhibition of 11 hydroxylase by metopirone. This phenomenon revealed that this patient might have sufficient 11 hydroxylase and a deficiency of 21 hydroxylase. The administration of dexamethasone made excretion of urinary steroids decrease. During treatment (cortisol 45 mg/day) the urinary steroid pattern became similar to that of a normal male and there were glucocorticoid metabolites present (THE, THF etc.) but still moderately increased pregnanetriol and pregnanetetrol. The other 2 cases of 21 hydroxylase deficiency showed very similar patterns to case 1. and were easily diagnosed. In the pregnant female, as stated by R. Rivera et al. there were unanalyzable peaks present near the pregnanediol peak. These other peaks could represent isomers of pregnanediol or other C-21 steroid metabolites which may be excreted in increasing amounts as pregnancy advances. Fig. 3 and 4 showed increased metabolites of progesterone. Sheehan's disease showed markedly decreased urinary steroids (Fig. 15). Primary hypothyroidism (Fig. 16 and 17) showed decreased urinary steroid excretion, which might be due to secondary hypopituitalism because they responded well to ACTH stimulation. The ability to perform these assays in the same aliquot of urine using this simultaneous enzymatic hydrolysis and extraction followed by acid hydrolysis and double internal standard technique reduces considerably the difficulty of identification of steroids and the time cosumed for the quantitation of

these substances by other procedures. It is suggested that this method may well be the preferred and the most useful method for clinical assay.

SUMMARY

A modified method with a new hydrolysis technique, simultaneous enzymatic hydrolysis and extraction and with a double internal standards technique was described. This method enabled to shorten the incubation time and to identify the analyzed steroid peaks more easily, and contributed to the presentation of several sets of clinical data which produced the valuable informations for the clinicians. Discussion about the advantage of this method for the routine study was made.

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