Journal of Chromatography, 374 (1986) 5—16
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2823

SIMULTANEOUS QUANTITATIVE MEASUREMENT OF FOURTEEN ADRENAL STEROIDS BY CAPILLARY COLUMN GAS CHROMATOGRAPHY—MASS SPECTROMETRY, AND ITS CLINICAL APPLICATION

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(First received November 14th, 1984; revised manuscript received July 23rd, 1985)

SUMMARY

Until now, there has been little work covering all of the main native adrenal-cortical steroids in blood. We therefore established a method for the simultaneous quantitative measurement of 14 native adrenal-cortical steroids, which involves capillary column gas chromatography—mass spectrometry (GC—MS). Serum steroids were purified from serum with the Extrelut® mini-column and then converted into stable derivatives for GC—MS by a combination of boronic cyclization and trimethylsilyl and methyloxime derivatization.

0378-4347/86/$03.50

The sensitivities (with a signal-to-noise ratio ≥ 7) of our GC—MS method ranged from 0.1 to 1.0 ng/ml of serum, and the coefficients of variation of intra- and inter-assays were < 19% for each steroid. Our newly devised method involving a capillary column GC—MS system has been proven to be a simple and suitable method for a diagnosis requiring simultaneous detection of many native adrenal steroids in clinical practice. The analysis time is only 4 h.

INTRODUCTION

For the purposes of diagnosing and following up adrenal disorders, we have developed a method for simultaneous measurement of 17-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, dehydroepiandrosterone, desoxycorticosterone, oestrone, androstenedione, oestradiol, pregnenolone, testosterone, progesterone, oestriol, corticosterone and cortisol. These fourteen serum steroids are important for the diagnosis of adrenal disorders such as 21-hydroxylase deficiency [1, 2]. There have been many reports of steroid measurement by gas chromatography—mass spectrometry (GC—MS) [3—14]. However, there have been few reports covering all of the main native adrenal-cortical steroids in blood. The reasons for this are supposed to be the lower concentrations in blood, and the difficulty of derivatizing corticosteroids [15, 16]. Our assay method reported here solved these problems with the Extrelut® column extraction for purification and *n*-butylboronic cyclization combined with ordinary trimethylsilyl and methyloxime (TMS—MO) derivatization [17] for better separation of the main native steroids.

This improved sensitivity made our assay method more simple and time-saving compared to other ordinary assay systems such as radioimmunoassay (RIA) [18, 19]. By applying this method, we were even able to distinguish and control the subtle types of congenital adrenal hyperplasia (CAH) in patients. For accurate diagnosis and follow-up in patients with adrenal disorders such as CAH, several abnormal adrenal steroids have to be measured and these abnormalities are the best indication for control. Radioimmunoassays have the disadvantage of cross-reactivity with other steroids, which is liable to prevent correct evaluation of adrenal disorders such as CAH with unknown abnormal steroid levels [20, 21]. With radioimmunoassays, moreover, a long analysis time and multiple assays are required to determine many types of steroids, because one RIA system cannot evaluate two types of steroid. Therefore, our newly devised method using a capillary column GC—MS system has been shown to be a simple and suitable method for a diagnosis requiring simultaneous detection of many native adrenal steroids in clinical practice.

EXPERIMENTAL

Chemicals

All steroids were purchased from Sigma (St. Louis, MO, U.S.A.). Trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI), N,O-bis(trimethyl-

silyl)trifluoroacetamide (BSTFA), *n*-butylboronic acid and methoxyamine hydrochloride were purchased from Gasukuro Kogyo (Tokyo, Japan). All solvents used were of the highest analytical grade. An Extrelut column, filled with granular Kieselguhr, was obtained from Merck (Darmstadt, F.R.G.).

Isolation and derivatization

Fig. 1 shows the procedures of extraction, isolation and derivatization of steroids for this assay. Blood samples were collected before and 60 min after intravenous administration of adrenocorticotropin hormone (ACTH) from four patients with 21-hydroxylase deficiency treated with hydrocortisone. After collection of blood, the serum was separated by centrifugation at 1000 *g* and at 4°C. Serum (1 ml) was mixed with 10 ng of 5α-cholestane as an internal standard and then the mixture was transferred to the Extrelut mini-column. After distribution of the aqueous phase on the column, steroids were twice-eluted with dichloromethane from the mini-column. The eluate with dichloromethane was evaporated at 60°C under a stream of nitrogen. Then, 100 μl of 10% *n*-butylboronic acid in ethyl acetate (10%, w/w) was added. The vials containing the reaction mixtures were agitated for 10—15 min at room temperature. The steroids possessing hydroxy groups at both the C-21 and C-17 positions were esterified with *n*-butylboronic acid to form cyclic boronate derivatives. The solvent was then evaporated under a stream of nitrogen. A

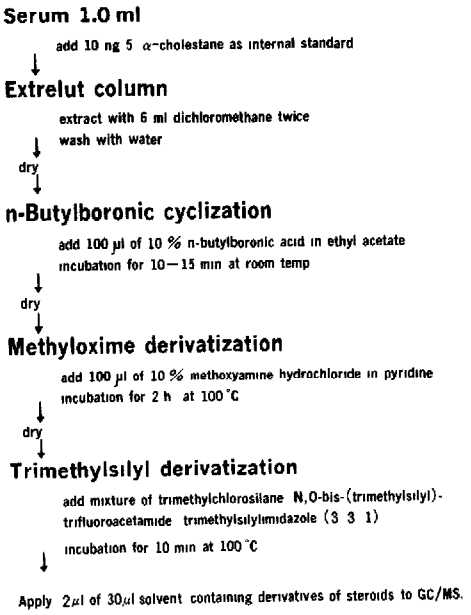

Fig. 1. The procedures of extraction, isolation, derivatization and measurement of fourteen serum steroids from blood samples

100-μl aliquot of 10% methoxyamine hydrochloride in pyridine (10%, w/w) was added. The mixtures were incubated for 2 h at 100°C, and then the solvent was evaporated. The steroids possessing carbonyl groups were converted to MO derivatives by reaction with methoxyamine hydrochloride. A 100-μl aliquot of a freshly prepared mixture of TMCS, BSTFA and TMSI (3:3:1, v/v/v) was added to the residue to obtain the TMS ether derivatives of the steroids. The residues were evaporated under a stream of nitrogen, and then dissolved in 30 μl of cyclohexane. A 2-μl aliquot of the 30 μl of solvent containing derivatives of steroids was subjected to GC—MS.

Capillary gas chromatography—mass spectrometry

A JMS-DX300, MS-GCG05 and JMA-2000H (gas chromatograph—mass spectrometer—computer combined system, Jeol, Tokyo, Japan) were used. A capillary column (cross-linked methyl silicone film, 25 m × 0.2 mm I.D., 0.11 μm thin film, Hewlett-Packard, U.S.A.) was used for the gas chromatographic step. Helium was used as the carrier gas at a flow-rate of 1 ml/min. The column temperature was set at 100°C and increased at a rate of 32°C/min from 100 to 200°C and at a rate of 8°C/min from 200 to 290°C. The ionization energy was 70 eV, the ionization current 300 μA, and the accelerating voltage 3 kV. The mass spectrometer was focused successively on the molecular ions of each steroid in the range from *m/z* 344 to *m/z* 558.

RESULTS

The recoveries of the steroids after Extrelut mini-column extraction are shown in Table I. Fig. 2 shows the structures of the derivatives of the steroids. These derivatives were formed by the combination of boronic cyclization and TMS—MO derivatization. We confirmed the detection of single peaks of individual steroids on gas chromatography.

TABLE I

RECOVERIES OF STEROIDS WITH THE EXTRELUT COLUMN

The data show mean recoveries (± S.D.) of fourteen ^{3}H-labelled radioactive steroids added to 1 ml of sterum on Extrelut mini-column extraction with dichloromethane (n = 6).

Steroid	Recovery (%)
Dehydroepiandrosterone	78.3 ± 5.4
Oestrone	89.9 ± 4.3
Androstenedione	97.7 ± 3.4
Oestradiol	92.8 ± 6.3
Pregnenolone	71.4 ± 3.4
Testosterone	89.8 ± 5.3
Progesterone	71.7 ± 3.2
Oestriol	83.9 ± 2.8
17-Hydroxyprogesterone	93.1 ± 3.4
Desoxycorticosterone	84.0 ± 5.7
Corticosterone	100.0 ± 0.0
11-Deoxycortisol	78.6 ± 3.3
21-Deoxycortisol	80.2 ± 6.1
Cortisol	76.3 ± 4.6

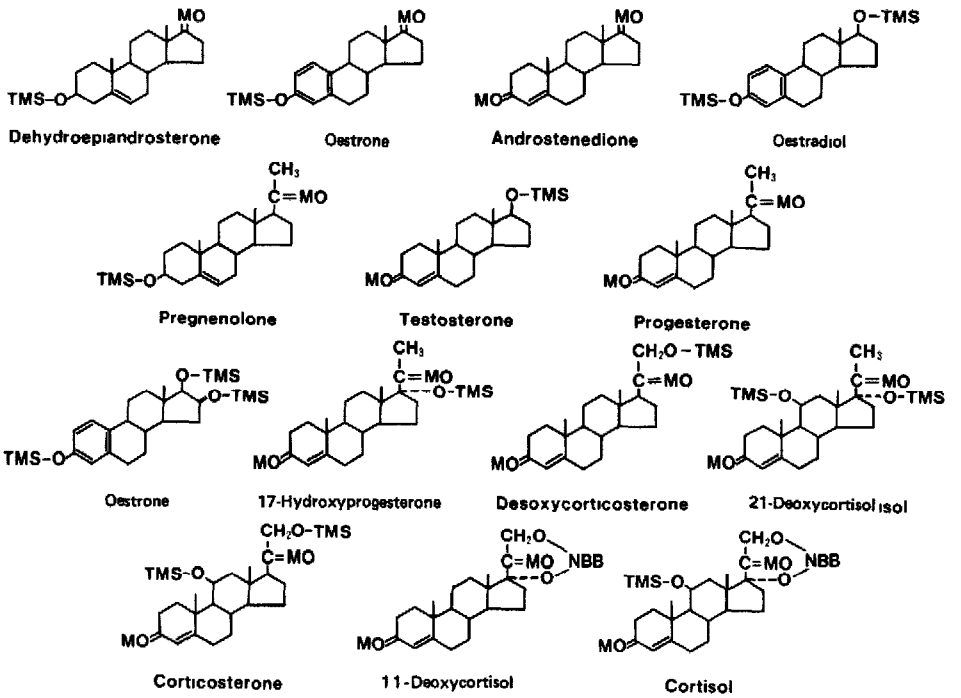

Fig. 2. Structures of the derivatives of steroids for application to GC—MS. NBB = n-C_4H_9; MO = CH_3ON; TMS = $(CH_3)_3Si$.

The structures of all the derivatives differed from each other. The mass spectra of the steroid derivatives are shown in Fig. 3. Since the molecular ions were strong enough to be used for measurement of all steroids and were more specific to individual steroids than other peaks, the molecular ions were used for quantitation as far as possible. However, for quantitative analysis of 17-OH-progesterone and dehydroepiandrosterone, characteristic strong ions (m/z 429 and m/z 358, respectively) were also used in addition to the molecular ions, because the intensities of these ions were stronger than those of the molecular ion peaks. Fig. 4 shows the gas chromatographic profiles of the derivatives of each steroid. Fig. 5 shows a mass fragmentogram of the molecular ions of the steroid derivatives extracted from serum samples that were fortified with 10 ng of each steroid and 2 ng of 5α-cholestane before purification. Oestradiol and pregnenolone were not separated by the capillary column, but they could be separated on the mass fragmentogram. The standard curves of the steroids are shown in Fig. 6. These standard curves were drawn on the basis of the ratios between the peak areas of the molecular ions of the steroid derivatives and that of 10 ng of 5α-cholestane. These standard curves were approximately linear from each lower limit level to 20 ng/ml of serum (shown in Fig. 6). Table II shows the sensitivities and recoveries with this assay. The sensitivities for steroids (signal-to-noise ratio $\geqslant$ 7) with this assay method ranged from 0.1 to 1 ng/ml of serum, which shows that it can be used to assess physiological levels of individual steroids. Coefficients of variation at detectable

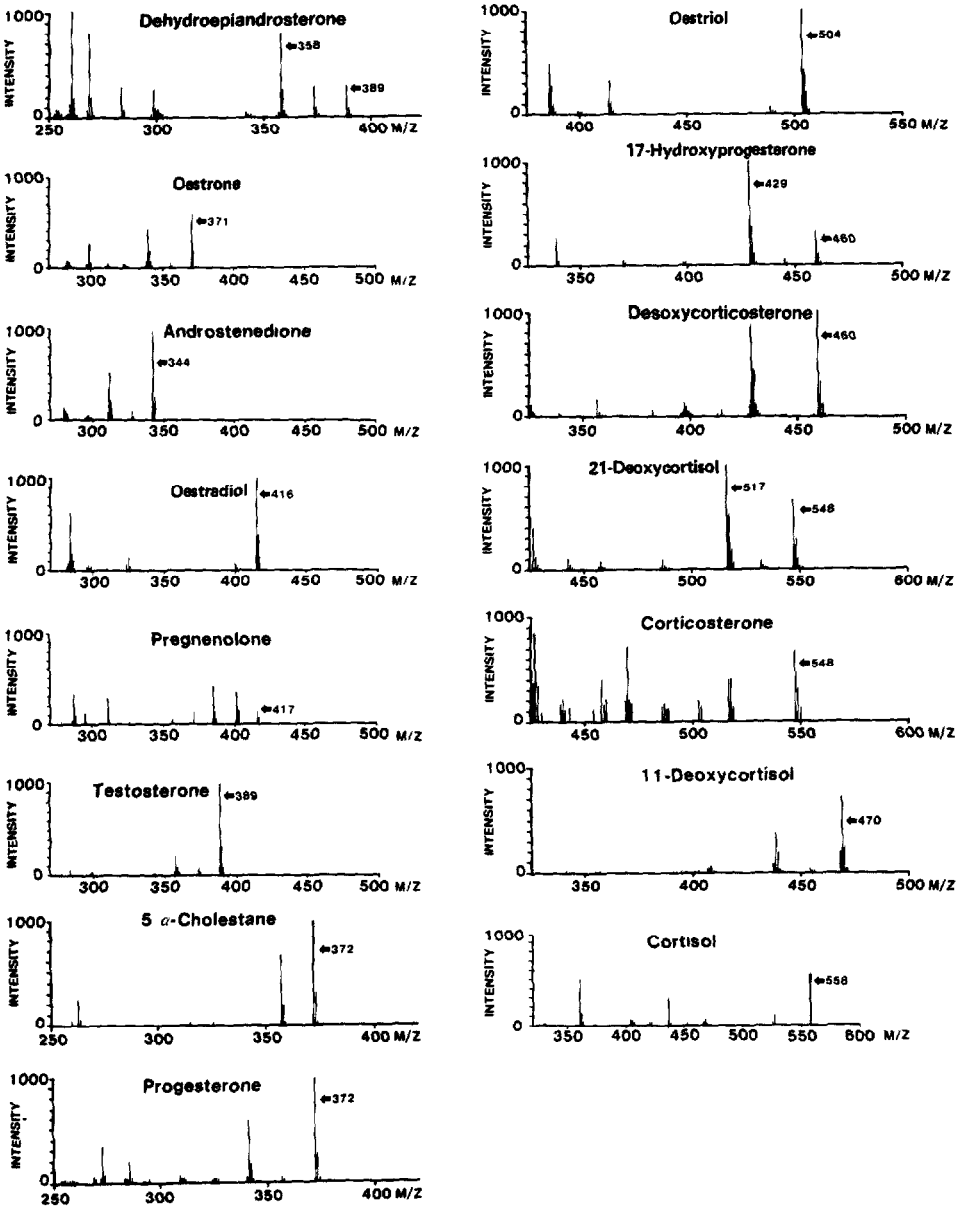

Fig. 3. Mass spectra of derivatives of steroids. Molecular ions of derivatives of the steroids are as follows: *m/z* 389 for dehydroepiandrosterone, *m/z* 371 for oestrone, *m/z* 344 for androstenedione, *m/z* 416 for oestradiol, *m/z* 417 for pregnenolone, *m/z* 389 for testosterone, *m/z* 372 for 5α-cholestane, *m/z* 372 for progesterone, *m/z* 504 for oestriol, *m/z* 460 for 17-hydroxyprogesterone, *m/z* 460 for desoxycorticosterone, *m/z* 548 for corticosterone and 21-deoxycortisol, *m/z* 470 for 11-deoxycortisol and *m/z* 558 for cortisol.

levels (signal-to-noise ratio ⩾ 7) were 6.5—19.0%. Fig. 7 shows the abnormal features of the steroid profile in a mass fragmentogram for a patient (a 7-year-old boy) with CAH due to 21-hydroxylase deficiency; these abnormal features were valuable for the diagnosis of this adrenal disorder. It is a

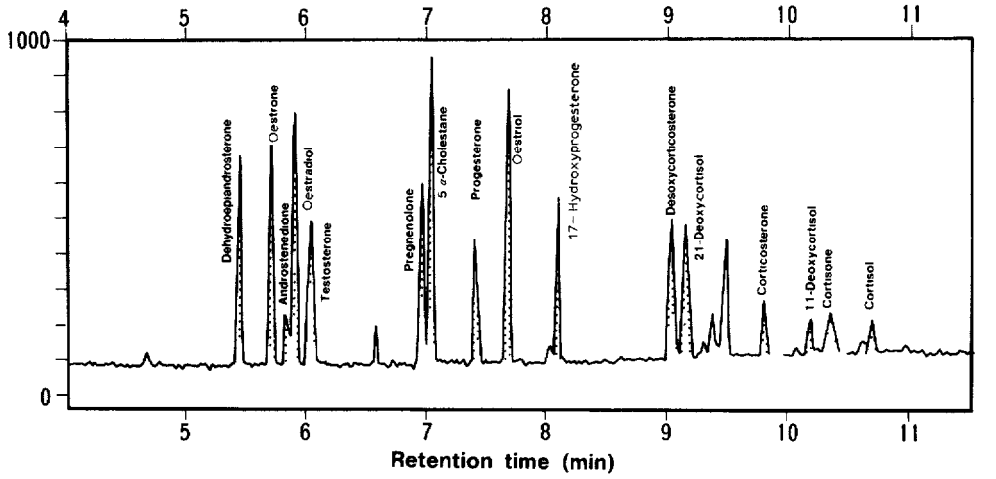

Fig. 4. Capillary gas chromatogram of the derivatives of standard steroids.

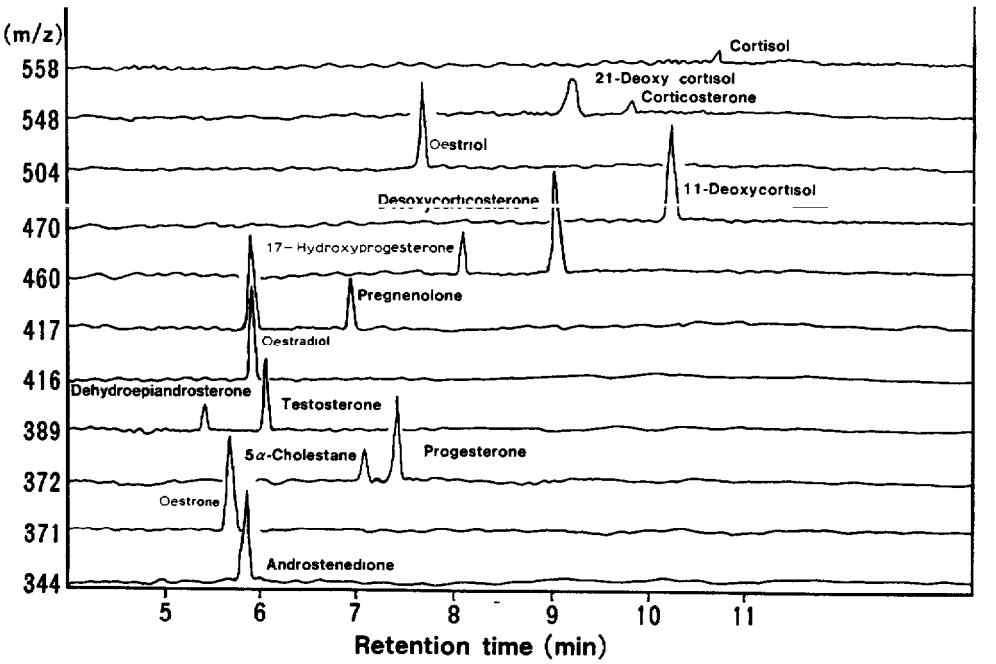

Fig. 5. Mass chromatogram of the derivatives of standard steroids. The mass fragmentogram is based on the molecular ions of derivatives of steroids extracted from serum samples which were fortified with 10 ng of each pure steroid before purification.

statistical fact that patients with 21-hydroxylase deficiency occupy more than 80% of child adrenal disorder cases. Table III shows the serum steroid levels calculated by the GC—MS method before and 60 min after intravenous administration of ACTH to four patients with 21-hydroxylase deficiency treated with hydrocortisone for evaluating the reserve of their adrenal glands. Serum corticosterone concentrations increased a little in case 2 and much more in cases 1, 3 and 4. The former was suspected of being of the salt-losing type, which was confirmed by the clinical symptoms. On the other hand, the latter

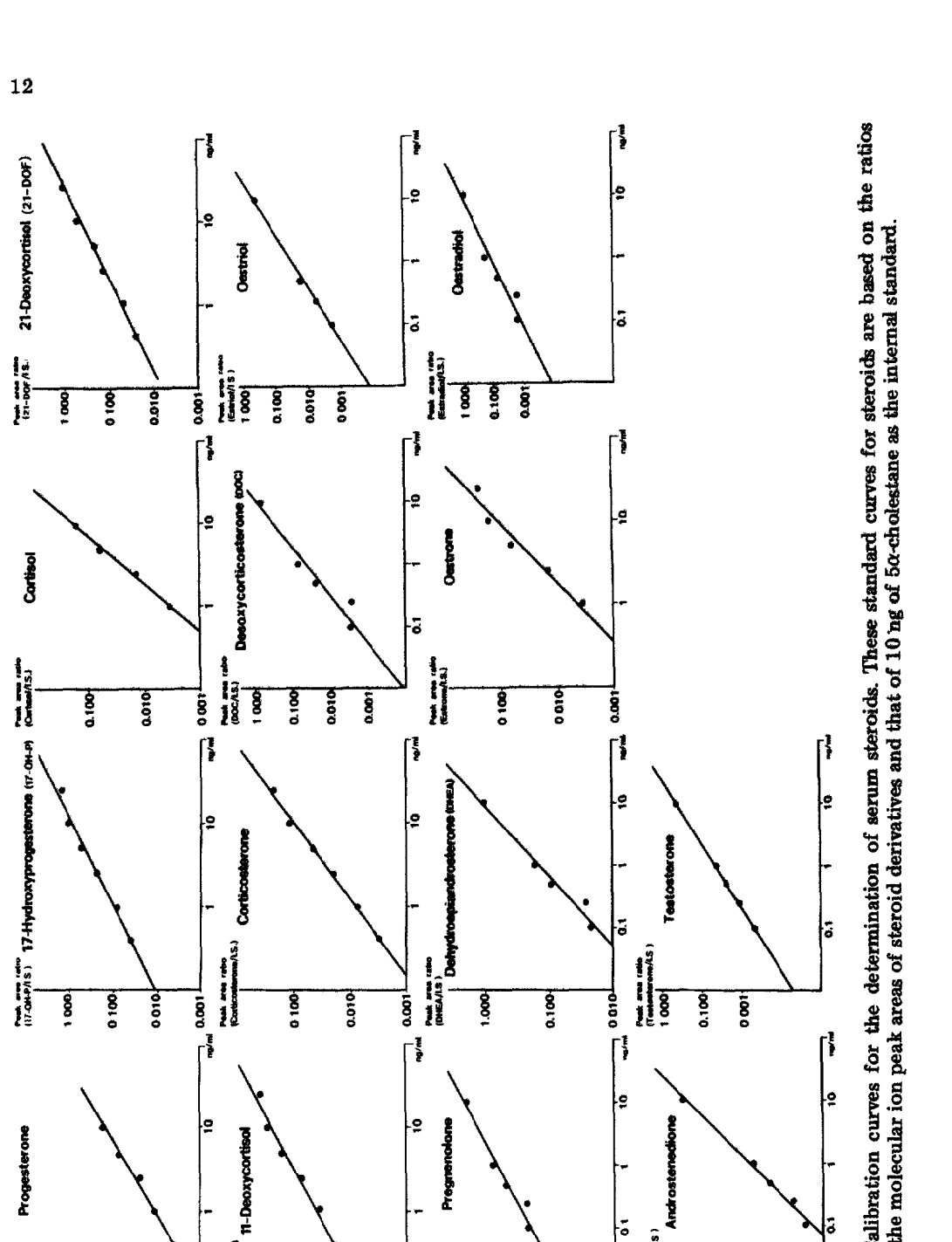

Fig. 6. Calibration curves for the determination of serum steroids. These standard curves for steroids are based on the ratios between the molecular ion peak areas of steroid derivatives and that of 10 ng of 5α-cholestane as the internal standard.

TABLE II

SENSITIVITIES AND RECOVERIES AT DETECTABLE LEVELS WITH GC—MS ASSAY

Steroid	Lower limit of detection* (ng/ml)	Accuracy (n = 6) (%)	
		Intra-assay	Inter-assay
Dehydroepiandrosterone	0.2	7.5	9.5
Oestrone	1.0	6.5	9.0
Androstenedione	0.1	15.0	15.5
Oestradiol	0.1	9.5	10.5
Pregnenolone	0.2	10.5	14.0
Testosterone	0.1	8.0	11.0
Progesterone	0.5	6.5	7.0
Oestriol	0.1	8.0	11.5
17-Hydroxyprogesterone	0.2	5.0	6.0
Desoxycorticosterone	0.2	8.0	10.0
Corticosterone	0.5	12.5	13.0
11-Deoxycortisol	0.5	5.5	6.0
21-Deoxycortisol	0.5	7.5	8.5
Cortisol	1.0	18.0	19.0

*Signal-to-noise ratio ⩾ 7.

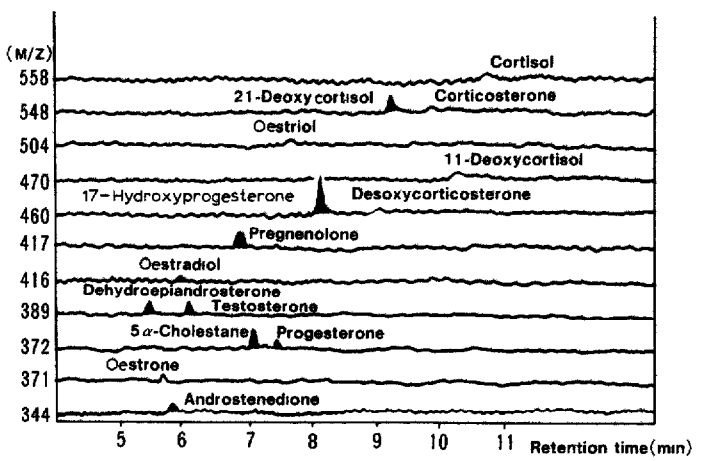

Fig. 7. Abnormal features of the steroid profile in a mass fragmentogram for a patient (a 7-year-old boy) with 21-hydroxylase deficiency treated with hydrocortisone.

were shown, finally, to be the simple type, as derived from their clinical course. Case 1 showed some response to deoxycorticosterone and corticosterone but the levels of the hormones were lower than in cases 3 and 4. This case was thought to be the simple type, because of the easy control with only a cortisol supplement; however, salt-losing shock developed at 7 years old and the patient regretfully expired. The patient might have always been insufficient in mineralocorticoid. We had the opportunity to obtain samples afterwards, and it may be possible to prevent death in these cases, if GC—MS becomes more popular. We emphasize that in this unfortunate case, the dangerous state of the patient under emergency conditions was not detected, because it was very expensive to measure all the steroid levels in each condition by RIA and it was

TABLE III

SERUM STEROID LEVELS IN PATIENTS WITH 21-HYDROXYLASE DEFICIENCY

The serum steroid levels were calculated by the GC—MS method before and 60 min after intravenous administration of ACTH to patients with 21-hydroxylase deficiency treated with hydrocortisone. Case 1 was a 7-year-old boy, case 2 a 7-year-old boy, case 3 a 13-year-old-girl and case 4 a 13-year-old boy. Oestrone, oestradiol and oestriol were undetectable in these cases.

Steroid	Steroid level (ng/ml)							
	Case 1		Case 2		Case 3		Case 4	
	Before	After	Before	After	Before	After	Before	After
Dehydroepiandrosterone	0.3	0.3	0.3	0.3	1.0	1.1	0.8	0.9
Androstenedione	0.1	0.2	0.1	0.2	1.7	2.4	0.8	0.8
Pregnenolone	1.1	2.7	0.4	0.4	2.1	2.2	1.2	4.2
Testosterone	0.2	0.3	0.2	0.3	0.5	0.6	6.8	6.9
Progesterone	4.2	24.5	2.2	4.2	12.0	18.0	13.4	21.1
17-Hydroxyprogesterone	12.8	41.3	14.7	23.5	74.0	91.8	34.2	50.4
Desoxycorticosterone	0.2	0.3	0.2	0.3	0.5	0.6	0.4	0.6
Corticosterone	0.7	2.0	2.0	2.3	4.4	5.5	3.8	5.4
11-Deoxycortisol	0.3	0.5	0.4	0.4	1.4	1.4	1.2	1.6
21-Deoxycortisol	2.1	3.8	3.6	4.8	14.8	17.8	16.0	21.2
Cortisol	22.5	72.8	26.4	34.6	162.0	173.0	145.0	163.0

also very time-consuming. These findings show that this assay system is useful not only for evaluation of adrenal control but also for assessment of enzyme deficiencies.

The responses of adrenal glands to ACTH loading, as shown in Table III, make it possible to determine the kind of enzyme deficiency.

DISCUSSION

For chemical diagnosis of adrenal disorders such as adrenal enzyme deficiencies [28], in which serum steroid levels are abnormally high or low, the analysis of serum steroids levels is required. At present, the measurement of serum adrenal steroids has been done mainly by RIA [18, 19, 22] and by high-performance liquid chromatography (HPLC) [10, 23]. Although RIA has the advantage of good sensitivity, multiple assay systems are required to obtain a steroid profile and complicated methods, such as Sephadex LH20 micro-column chromatography, are needed to avoid the cross-reactivities of antibodies. With HPLC, a serum steroid profile can be obtained easily; however, there is the problem of low sensitivity, making it difficult to apply to evaluations with blood samples, and many solvent systems are needed to improve the sensitivity. Thus, the GC—MS method is better and simpler. The sensitivities with GC—MS are higher than with HPLC, due to the easier mechanical purifications essentially using a millimass.

Even with these advantages of GC—MS methods, they are still difficult to apply to evaluations of blood adrenal steroids, due to contamination. This is one reason why there have been few reports so far concerning quantitative

evaluations; moreover, the majority of reports are concerned with evaluations of urine, due to the high concentrations involved [11—14]. Most of the reports on evaluations of blood steroids were on the assessment of a single steroid or only a few steroids [3—10]. Another factor is that strong silylation is needed to achieve the full silylation of adrenal steroids but, under these strong conditions, some steroids, such as cortisol, are easily decomposed [15, 16]. Thus, it is difficult to find universal conditions to assess all of the steroids simultaneously. The method described here involves the *n*-butylboronic cyclization in combination with the ordinary TMS—MO method [17], which makes it possible to separate several steroids and to stabilize the thermolabile steroids, such as cortisol. As described above, contamination by blood materials made it difficult to obtain high selectivities and sensitivities, which is one reason why there have been few reports on blood evaluations.

Recently, a method using an Extrelut column was described by Wehner and Handke [20]. Purification including this column chromatography makes it possible to assess practically all of the main native steroids in serum samples. With this assay, it takes only 4 h to obtain final results, which makes it possible to give speedy treatment and to get good control, especially in CAH patients. In the case of an adrenal enzyme deficiency in which the serum and urine steroid levels show an abnormal profile [12, 14, 23–28], interference due to cross-reactivity of unknown steroids should be considered when assessing the values obtained with a radioimmunoassay. On the other hand, there is no interference with our assay system involving the capillary column GC—MS method. By clinical evaluation with the ACTH loading test, detailed estimations [29, 30] were performed concerning the adrenal reserve and the severities of the disorders, and it was proved that our simplified method was useful not only for evaluation of adrenal disorders but also for speedy responses to the changing conditions.

CONCLUSIONS

(1) Our method makes it possible to analyse fourteen native adrenal steroids simultaneously.

(2) The sensitivities for each steroid range from 0.1 to 1 ng/ml (signal-to-noise ratio $\geqslant 7$).

(3) It only takes 4 h to analyse the fourteen native adrenal steroids with this assay.

(4) The easy measurement of adrenal steroids helps us to diagnose the type of enzyme deficiency and to make a speedy response to the changing conditions in adrenal disorders.

This method involving GC—MS with a capillary column, therefore proved to be available for the simultaneous measurement of serum steroids and thus to be useful for patients with adrenal disorders.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Mituru Osano (Department of Paediatrics) and Professor Ryuichi Kato (Department of Pharmacology, Keio University) for their interest and encouragement.

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