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RECENT ADVANCES IN DOPING ANALYSIS (3)

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Steroid Profiles, Disease and Gender Verification
-Inborn Errors of Metabolism-

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Urinary steroid profiles of neonatal and infant have been determined for the classification of steroid converting enzyme deficiency. Urine samples of the patient with 21-hydroxylase deficiency (Case-1), 17 α-hydroxylase deficiency (Case-2) and P-450 side chain cleavage enzyme (P-450scc) deficiency (Case-3) were analyzed. The major differences in the urinary steroid profiles of the patients were the excretion in much grater absolute and relative amount of: (1) 17 α-hydroxypregnenolone and 16 α-hydroxy dehydroepiandrosterone, (2) 5 α-tetrahydrocorticosterone (5 α-THB) respectively. 5 α-Tetrahydrocortisone (α-THE) and 20 β-cortolone were the only steroids excreted in the urine of the patient with P-450 scc deficiency before medical treatment(3), but the following application of cortisol (F) to the patient resulted in the urinary excretion of cortisol, cortisone and their tetrahydro metabolites. The results demonstrated that the patient with P-450 scc shows the presence of the other steroid converting enzyme activity, and the analytical results are informative for the research on the metabolism of corticosteroid. This report also re-validated the usefulness of steroid profiling on the positive case of gender verification.

Introduction

The classification of the congenital adrenocortical hyperplasia (CAH) using GC/MS is one of the important requirement in the routine urinalysis. The analytical procedure for the clinical diagnosis of CAH was developed by Shackleton[1)] and Axelson[2)]. Their methods consisted of deconjugation, extraction, methoxyme-TMS derivatization and GC/MS analysis. However, the presented reports in the literature focused on the individual case, and only limited information available that could be applied on the medical control in sports.

The several recent drug-testing incidents involved the doping cases with naturally occurring steroids. Further, some positive cases of gender verification may involve the patient with steroid converting enzyme deficiency and the athlete with transsexual

operation. Thus, the basic research of the steroid metabolism becomes more important also for maintaining the analytical procedure and the criteria for steroid doping.

Materials and methods

All the urine samples were collected from the neonatal and were analyzed for combined fraction steroids.

All solvents were of reagent grade or analytical grade. Methoxyamine hydrochloride and trimethylsilylimidazole were purchased from Wako pure chemicals (Tokyo). β-Glucuronidase/arylsulfatase form *Ampurallia* (Tokyo zouki) was used[3]. Sample preparation is shown in Figure-1.

MS conditions

Instrument:	Double focusing GC/MS type JMA-DX303 / DA5000 (JEOL, Tokyo)
Ionization	Electron impact (70 eV, 300 μ A)
Ion multiplyer	1.3 KV
Mass range	100 - 700 (m/z)
Scan rate	0.7 sec/scan
Ion source temp.	200°C

GC conditions

GC	MS-GC G06 (JEOL, Tokyo)	
Column	Methylsilicone OV-1, 0.243 mm ID x 30 m L 0.25 m μ film thickness(J&W,CA USA).	
Carrier gas	He, 1.85 kg/cm^3 (ca 1.5ml/min. at 100°C)	
Temperature	Injector:	300°C
	Column:	200-300°C (8°C/min) hold at 300°C for 10 min.
Split ratio	11:1	

Results and discussion

The major fragments of methoxyme-TMS steroids were M^+, M^+-15 (loss of methyl radical), M^+-90 (loss of trimethylsilyl alcohol radical in case of hydroxy steroids) and M^+-31 (loss of methoxy radical in case of ketosteroids). Some other characteristic fragments were also observed. The cleavage between C13-C17 of steroid skeleton results in the formation of D-ring fragment. D-ring fragment of 20 ζ-hydroxypregnanes and 21-hydroxypregnanes, or 17 hydroxy-20-ketopregnanes and 21-hydroxy-20-ketopregnanes give rise a signal of fragment ion (m/z) 117 $[C_2H_4OSi(CH_3)_3]^+$ or 188 $[C_4H_8Si(CH_3)_3NOCH^3]^+$ respectively. The major A-ring fragment of 3 ζ-hydroxy pregnane was 129 $[C_3H_4OSi(CH_3)_3]^+$. Molecular ion could hardly be detected in case

of non-keto poly hydroxy pregnanes, for example, pregnanediol, pregnanetriol, pregnenetriol and pregnanetetrol. Carbonyl group at C11 position of C-ring could not be derivatized into methyl oxyme and was detected as the native form. Some typical EI mass spectrums of methoxyme-TMS steroids were shown in Figure-2-1 to Figure-2-5.

Authentic steroids:

Standard mass chromatograms were given in Figure-3 and Figure-4. About 40 important endogenous steroids can be monitored simultaneously over one GC/MS analysis. Syn- and anti-isomers of dehydroepiandrosterone(DHEA), corticosteroids and some other ketosteroids were formed by methoxyme-TMS derivatization, and were detected as coupled peaks. The relative retention time and the EI mass spectra of authentic methoxyme-TMS steroids were in good agreement of those published in the literature (Table-1).

Steroid profiles in urine of normal infants:

The major difference in the urinary steroid profiles between newborn and normal adult is the increased excretion of 3 β-hydroxy-5-ene steroids. Some unknown isomers of 16-hydroxydehydroepiandrosterone, namely androst-ene-diol-mono-one, could be detected. Elevated levels of 16 α-hydroxylase activity is also expected. The concentration of 17 α-hydroxyprogesterone (17-OHP), a typical neonatal steroid, is highly correlated to the body weight and gestational age of newborn. In mature infants, the concentration of neonatal steroids fell rapidly soon after birth, and the period was around first 33 weeks of the life after gestation.[4)]

Case-1 CAH:	**21-Hydroxylase deficiency (Figure-5)**
Social sex (external genitalia type):	Male
Sex chromatin:	XX Female

The urine sample was collected on 6th. day after birth. The steroid profiles represented the large increase of the urinary absolute excretion of 16 α-hydroxy pregnenolone, DHEA, and 16 α-hydroxy DHEA. Concentration of pregnanetriol(P3) and pregnantriolone(11K-P3) were relatively higher than those of normal infants. Absence of 21-hydroxylase activity resulted in the suppressed urinary excretion of 5 α-THE, 20 β-Cortolone. It was expected that external expression of sexuality in this case was regulated by the elevated endogenous production of androgens.

Case-2 CAH: **17 α-Hydroxylase deficiency (Figure-6)**

Social sex (female type external genitalia): Female castrated

Sex chromatin: XY Male

The urinary steroid profiles of the patient were measured at one year after delivery. Because neonatal steroids still seen in the urine sample of this patient at one year old, this case was firstly expected as 3 β-hydroxy steroid dehydrogenase deficiency. Elevated level of total 17-hydroxycorticosteroid (17-OHCS) was measured by colorimetry, but the value was not in good agreement with the summation of 17-OHCS fractionation determined by GC-FID. Confirmation analysis by GC/MS was requested because the major unknown peak of GC chromatogram had the same retention time as those of 5β-tetrahydro cortisol (5β-THF) and the result was not agree with the other test results.

It was confirmed by MS analysis with modified GC temperature program that the main peak of the chromatogram was any of the isomer of pregnantriolones, and the peak was eluted slightly faster than 5 β-THF. By comparison with authentic pregnantriolones, the unknown peak was finally assigned as 5 α-THB. Thus, the elevated level of total 17-OHCS was validated. Major significant difference of the steroid profiles were the excretion in much grater abusolute amount of 5 α-THB, and relative amount of β-THA and 16α-hydroxypregnenolone. Suppressed excretion of 3 β-hydroxysteroids (DHEA and 16 α-hydroxy DHEA for example) was not caused by the deficiency of 3-hydroxysteroid dehydrogenase but caused by the lack of their parent compound.

Case-3 Prader's syndrome: **P-450 scc deficiency (Figure-7)**

Social sex (female type external genitalia): Female castrated

Sex chromatin: XY Male

Age: one year old

No significant amount of urinary steroids, except cholesterol exogenously taken from foods could be seen in the urine sample of the patient with P-450 scc deficiency. An additional urine specimen was also collected after single dose oral application of cortisol (F). Parent compound (F) and the metabolites e.g. cortisone(E), THF and THE were detected in the urine sample that collected after administration of F. This results indicated the presence of the other steroid converting enzymes. Since no biological background of E and F presents in this case, the metabolites of exogenous F could be identified easily. The proposed metabolic pathway of the neonatal steroids was summarized in Figure-8.

Expression of external sexuality in case-1 was regulated by the elevated endogenous production of androgens. On the other hand, suppressed maturity of sexual organ in case-2 and case-3 was due to the lack of androgens.

Conclusion

This report re-validated the usefullness of the traditional steroid profiling not only for doping control but also for the following up procedure of gender verification.

Acknowledgment

All the urine samples were collected by Dr. Keiko Honma at the Keio University Hospital School of Medicine, Endocrinology Department of Clinical Laboratories[5], and were analyzed in Tokyo laboratory.

References

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2) Analysis of Profiles of Conjugated Steroids in Urine by Ion-Exchange Separation and Gas Chromatography Mass Spectrometry. M.Alexson, B.L.Sahlberg and J.Sjoevall, *J.Chromatogr.* **224**, 355-270(1981)

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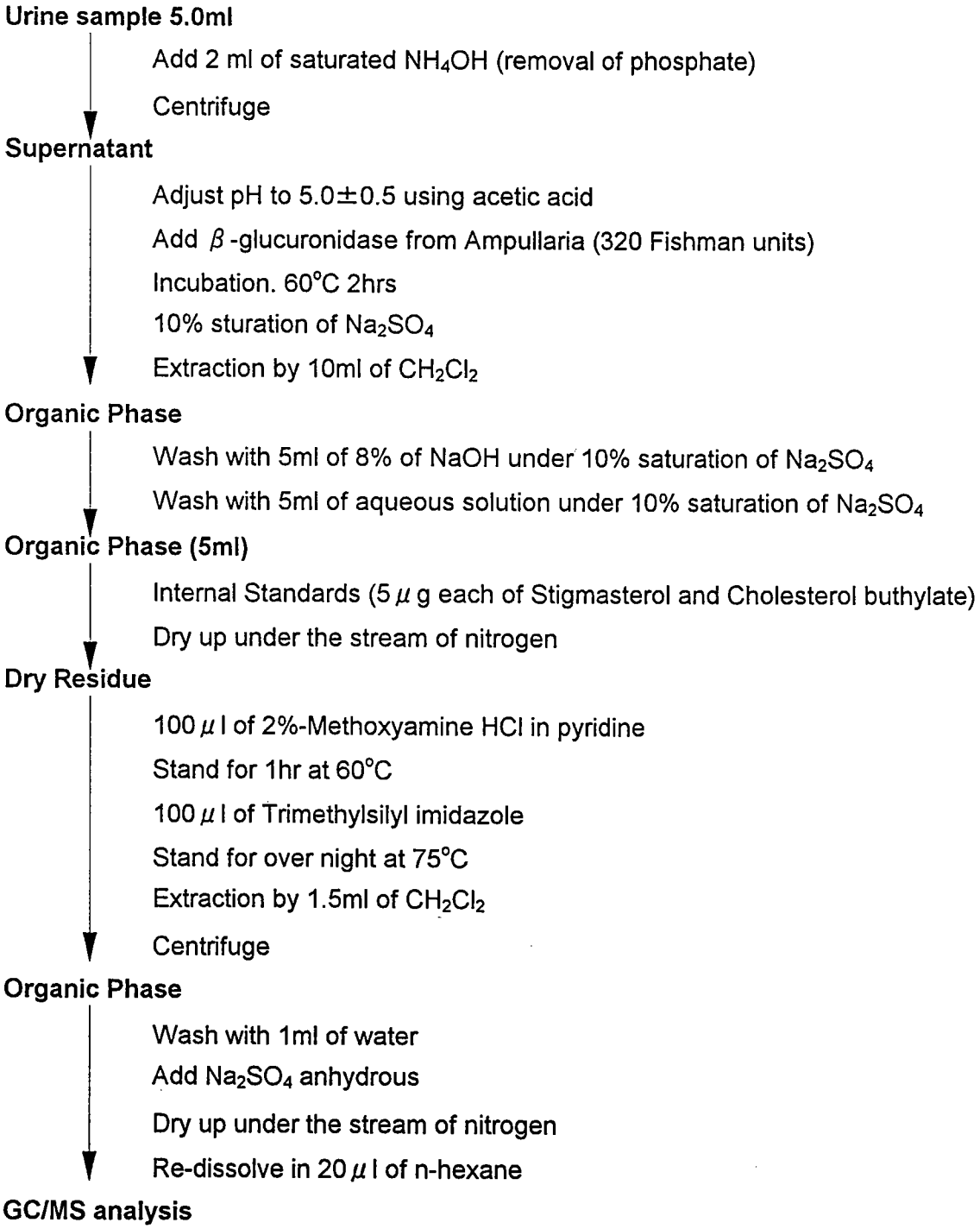
Table-1 The relative retention time and the monitoring ions of methoxyme-TMS steroids

Abbreviations	Summary Structure	Relative RT	MW	Monitoring Ions (M/Z)			
A	5α A-3α-ol-17-one	0.552	391	376	360	270	
E	5β A-3α-ol-17-one	0.559	391	376	360	270	
5α,3α-A2	5α A-3α,17β-ol	0.560	436	436	421	346	241
DHEA	A5en-3β-ol-17-one	0.591	389	389	358	260	
δ5-A2	A5en-3β,17β-ol	0.603	434	434	419	344	305
5α,3β-A2	5α A-3β,17β-ol	0.607	436	436	421	346	241
KA	5α A-3α-ol-11,17-one	0.609	405	405	374	315	300
KE	5β A-3α-ol-11,17-one	0.617	405	405	374	315	300
HA	5α A-3α,11β-ol-17-one	0.650	479	479	448	358	268
HE	5β A-3α,11β-ol-17-one	0.655	479	479	448	358	268
Pregnanolone	5β P-3α-ol-20-one	0.658	419	419	388	298	
16H-DHEA	A5en-3β,16α-ol-17-one	0.668	477	477	446	356	266
P2	5β P-3α,20α-ol	0.690	464	449	346	284	269
δ5-P	P5en-3β-ol-20-one	0.706	417	417	402	386	312
P3	5β P-3α,17α,20α-ol	0.706	552	552	435	345	255
TH-21-DOF	5β P-3α,11β,17α-ol-20-one	0.717	595	595	564	474	384
16K-δ5-A2	A5en-3β,17β-ol-16-one	0.719	477	477	462	446	356
δ5-P2	P5en-3β,20α-ol	0.726	462	462	447	372	
δ5-A3	A5en-3β,16α,17β-ol	0.727	522	522	432	342	329
β-THS	5β P-3α,17α,21-ol-20-one	0.732	595	595	564	492	474
δ5-17HP	P5en-3β,17α-ol-20-one	0.747	505	505	474	384	294
11K-P3	5β P-3β,17α,20α-ol-11-one	0.767	566	566	449	359	269
P4	5β P-3α,11β,17α,20α-ol	0.767	640	640	550	523	343
δ5-16HP	P5en-3β,16α-ol-20-one	0.780	505	490	474	384	188
β-THE	5β P-3α,17α,21-ol-11,20-one	0.788	609	609	578	488	398
δ5-P3	P5en-3β,17α,20α-ol	0.790	550	535	433	343	253
β-THA	5β P-3α,21-ol-11,20-one	0.797	521	521	490	431	400
β-THB	5β P-3α,11β,21-ol-20-one	0.802	595	595	564	474	384
δ5-3,16,20-P3	P5en-3β,16α,20α-ol	0.803	550	460	445		
δ5-21HP	P5en-3β,21-ol-20-one	0.810	505	505	474	402	239
α-THB	5α P-3α,11β,21-ol-20-one	0.814	595	595	564	474	384
β-THF	5β P-3α,11β,17α,21-ol-20-one	0.818	683	683	652	562	472
α-THF	5α P-3α,11β,17α,21-ol-20-one	0.823	683	683	652	562	472
20α-Cortolone	5β P-3α,17α,20α,21-ol-11-one	0.839	654	654	551	449	359
20β-Cortolone	5β P-3α,17α,20β,21-ol-11-one	0.859	654	654	551	449	359
Cholesterol	C5en-3β-ol	0.894	458	458	443	368	353
E	P4en-17α,21-ol-3,11,20-one	0.939	652	562	531	459	441
F	P4en-11β,17α,21-ol-3,20-one	0.990	636	636	605	515	361
20ζ-HP, 21-HP	[C2H4OSi(CH3)3]+			117	D-ring fragm.		
20K21HP, 20K17HP	[C4H6OSi(CH3)3NOCH3]+			188	D-ring fragm.		
3ζ-HP	[C3H4OSi(CH3)3]+			129	A-ring fragm.		
IS:Stigmasterol	S5,22dien-3β-ol	1.000	484	484	394	255	
IS:ButhylylChol	C5en-3β-O-(n-buthyl)ester	1.149	456	368	353	247	213

Abbriviations、A:Androstane, P:Pregnane, C:Cholestan, S:Stigmastane, A5en:5-Androsten
17H:17-Hydroxy, 11K:11-keto, TH:Tetrahydro

Figure-1 Sample preparation procedure for the steroid profiling



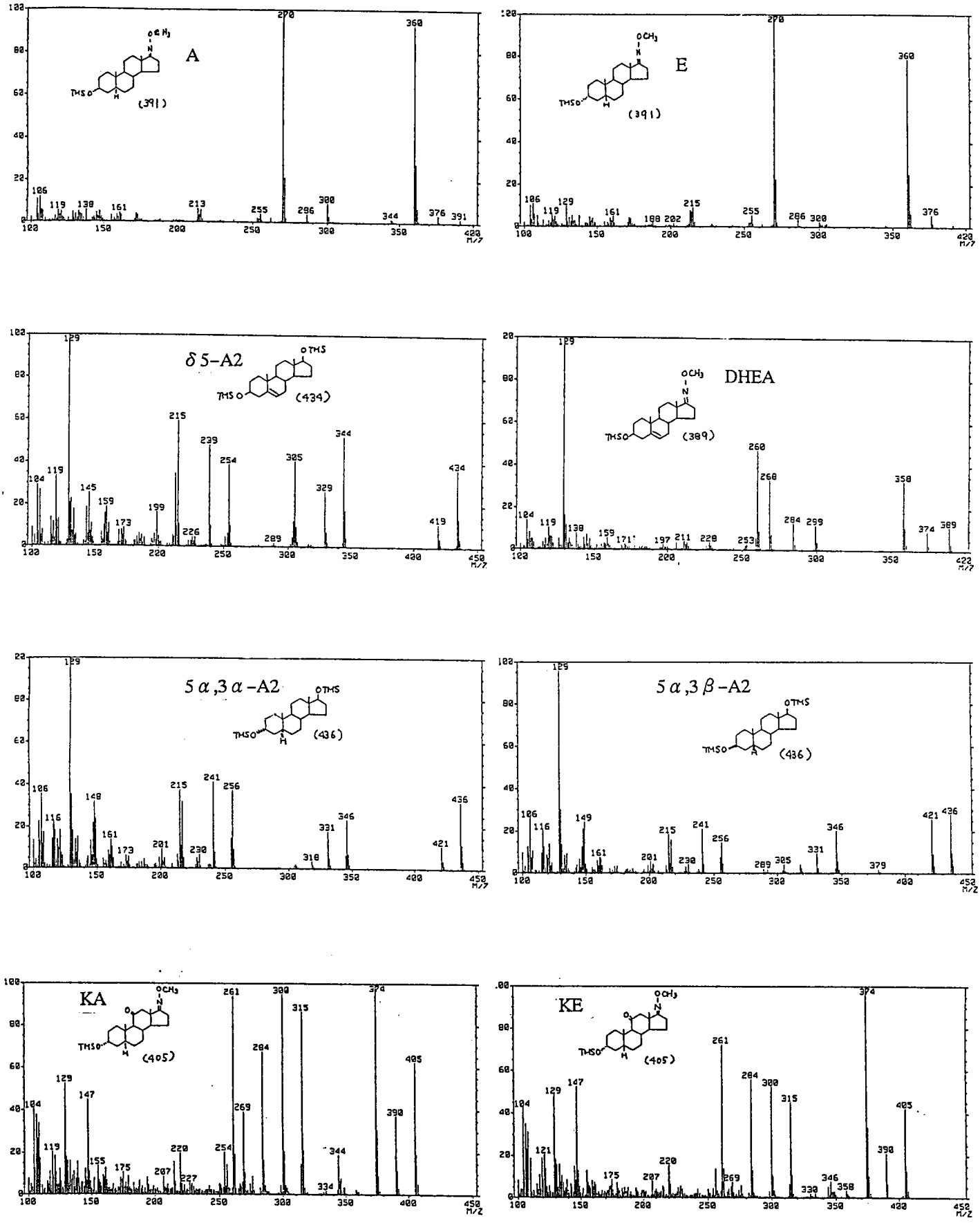


Figure-2-1 EI mass spectra of Methoxyme-TMS steroids.

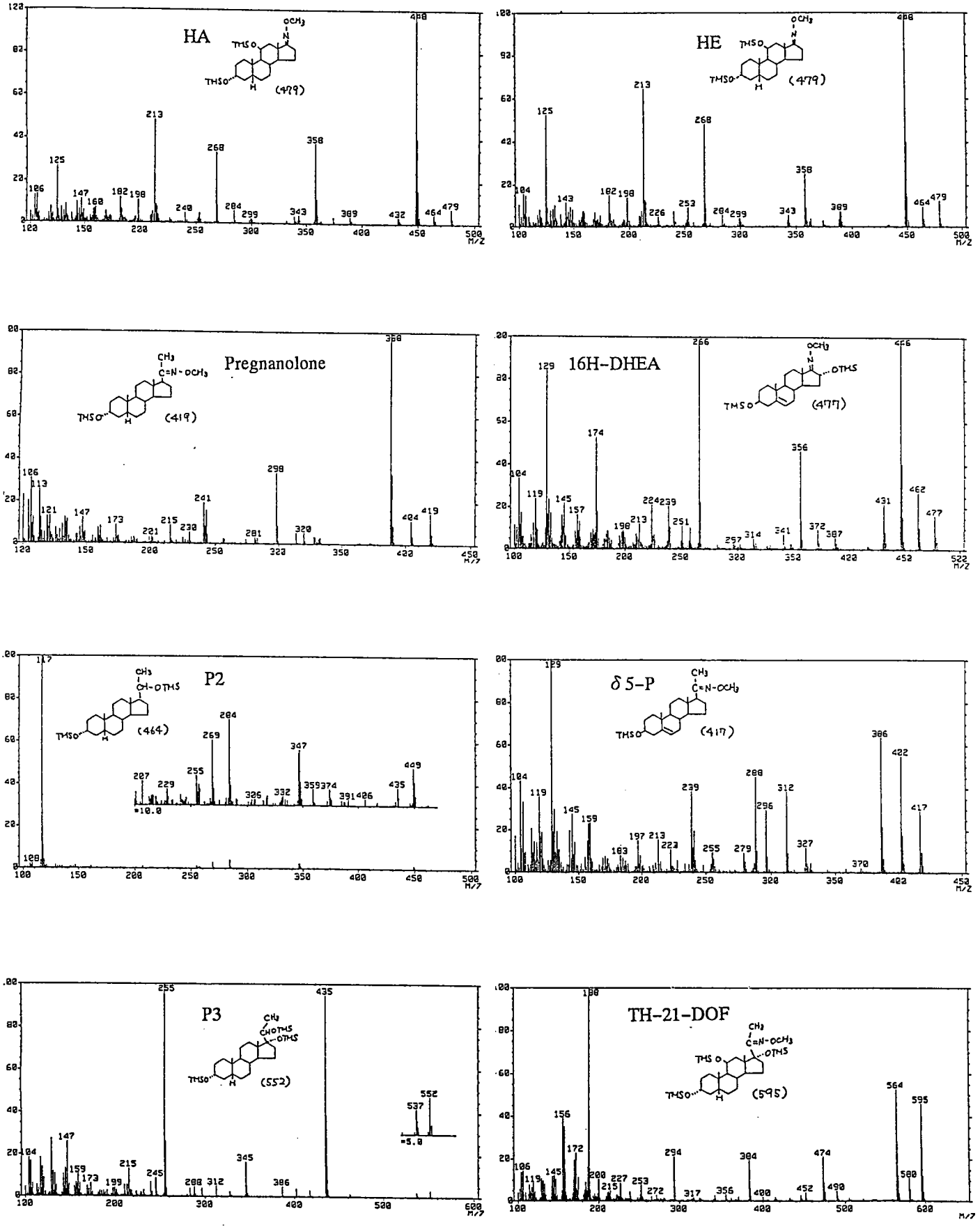


Figure-2-2 EI mass spectra of Methoxyme-TMS steroids.

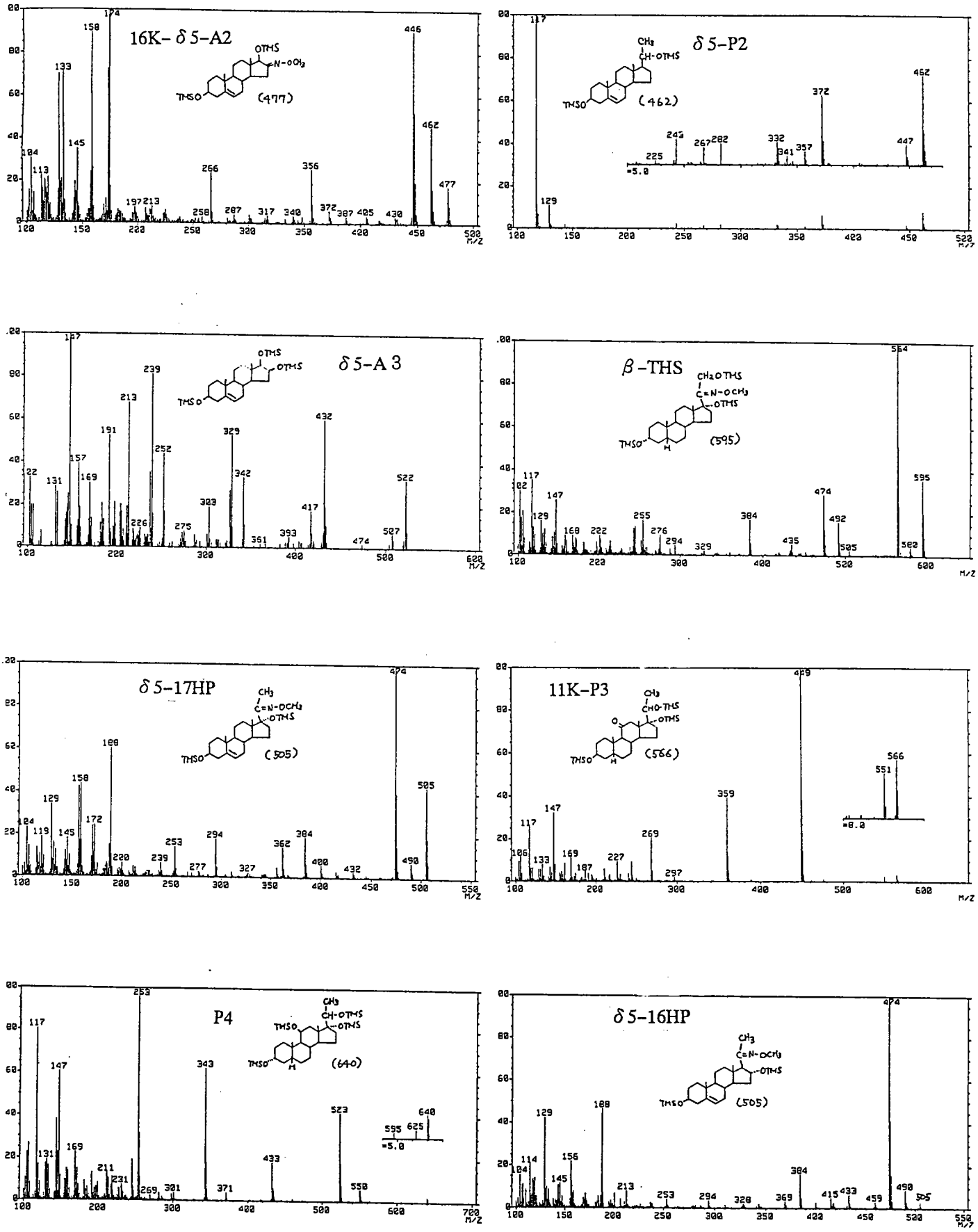
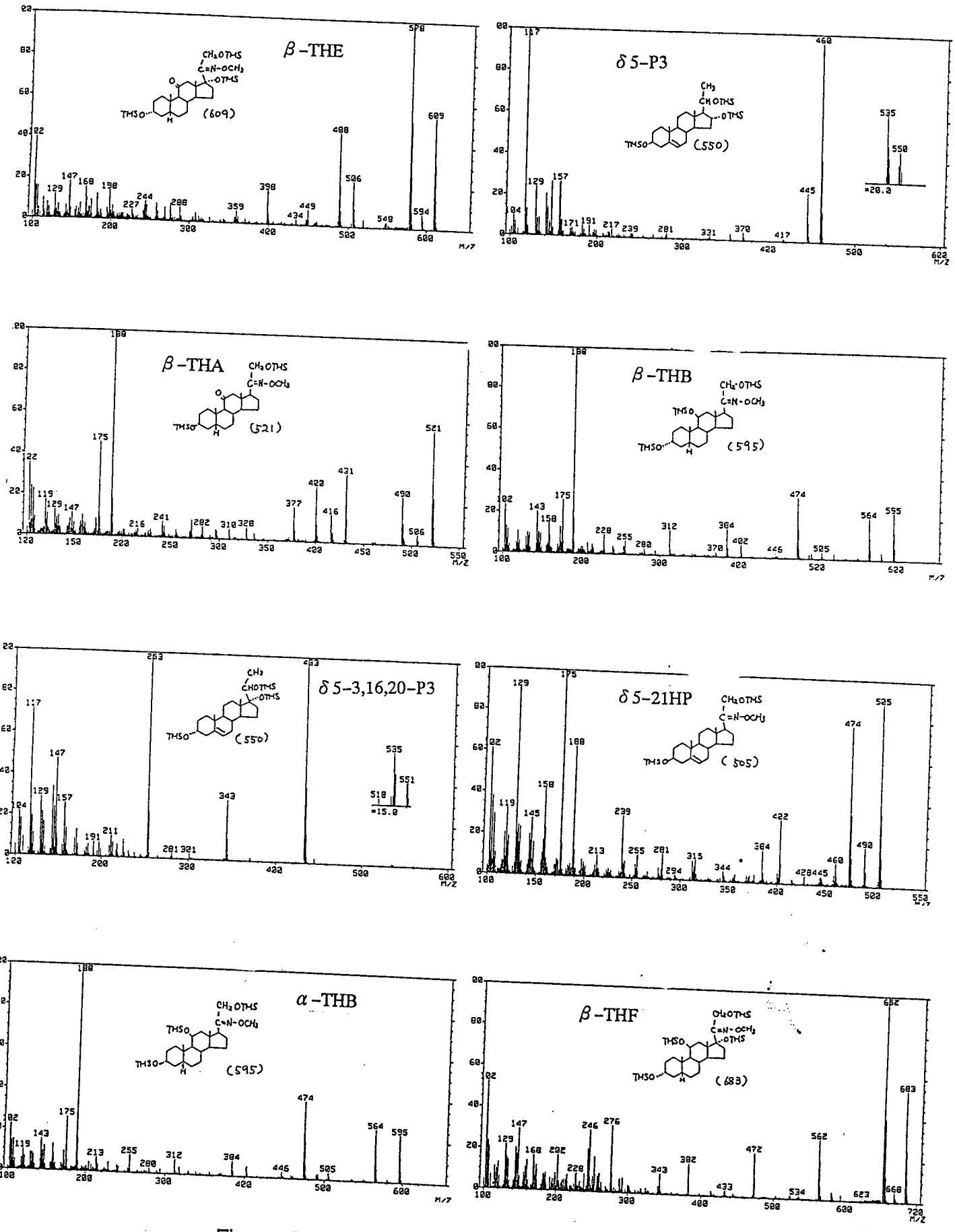
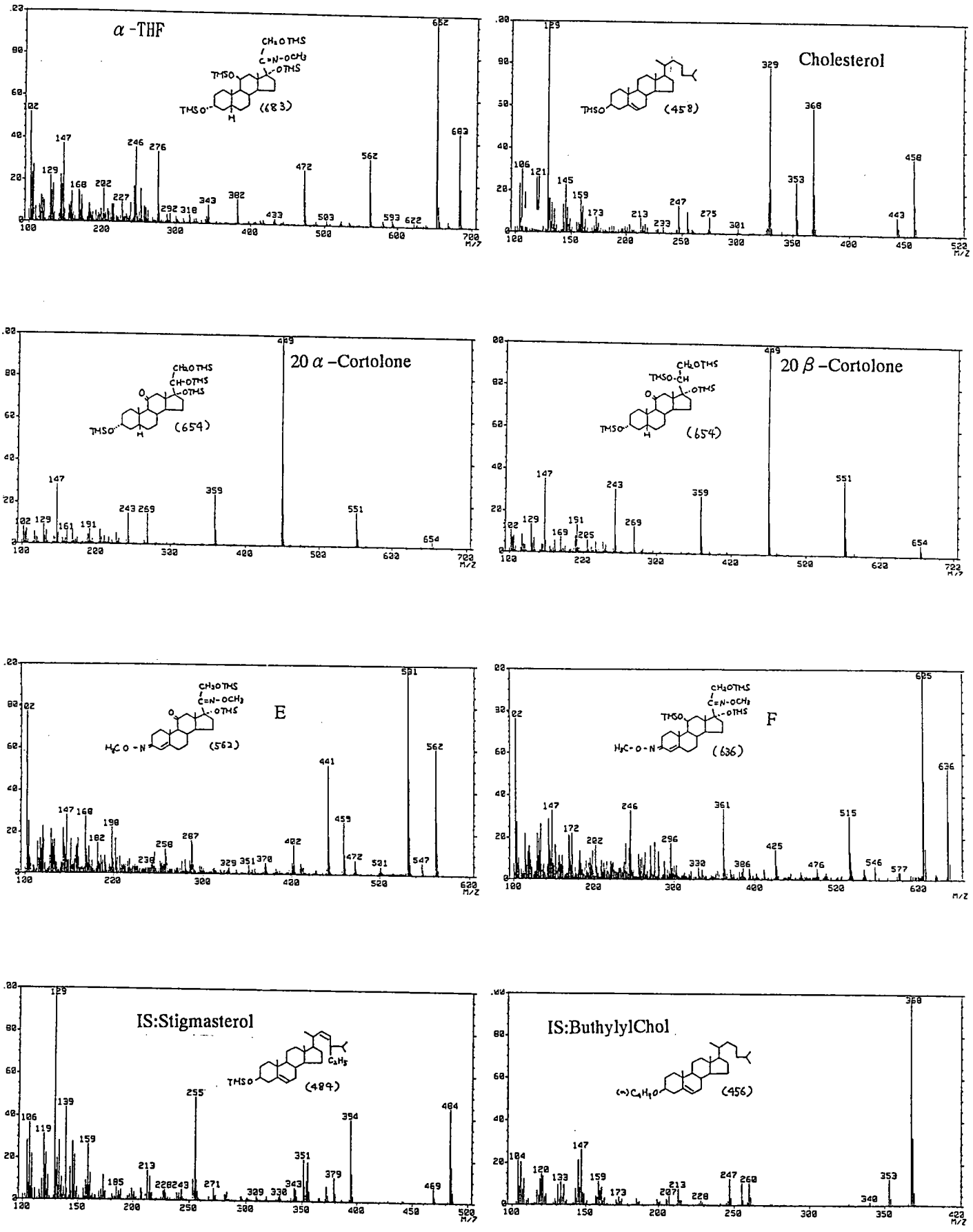


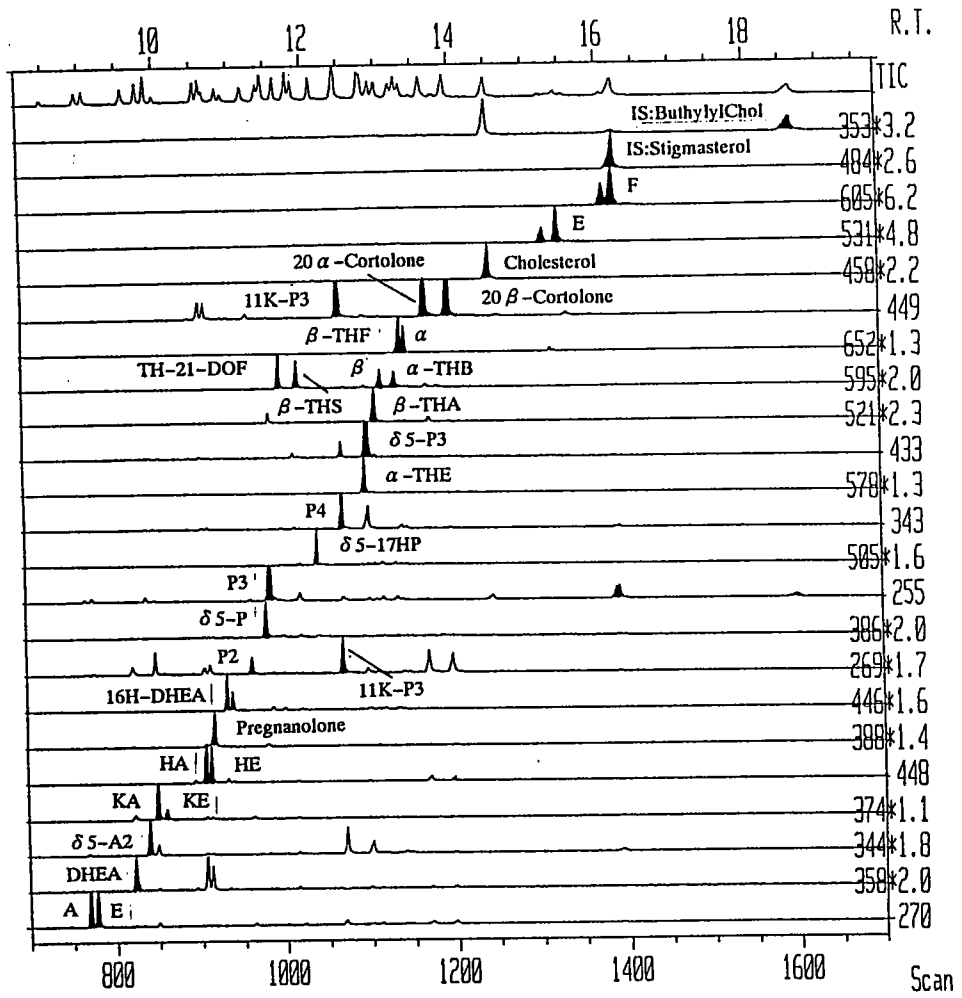
Figure-2-3 EI mass spectra of Methoxyme-TMS steroids.



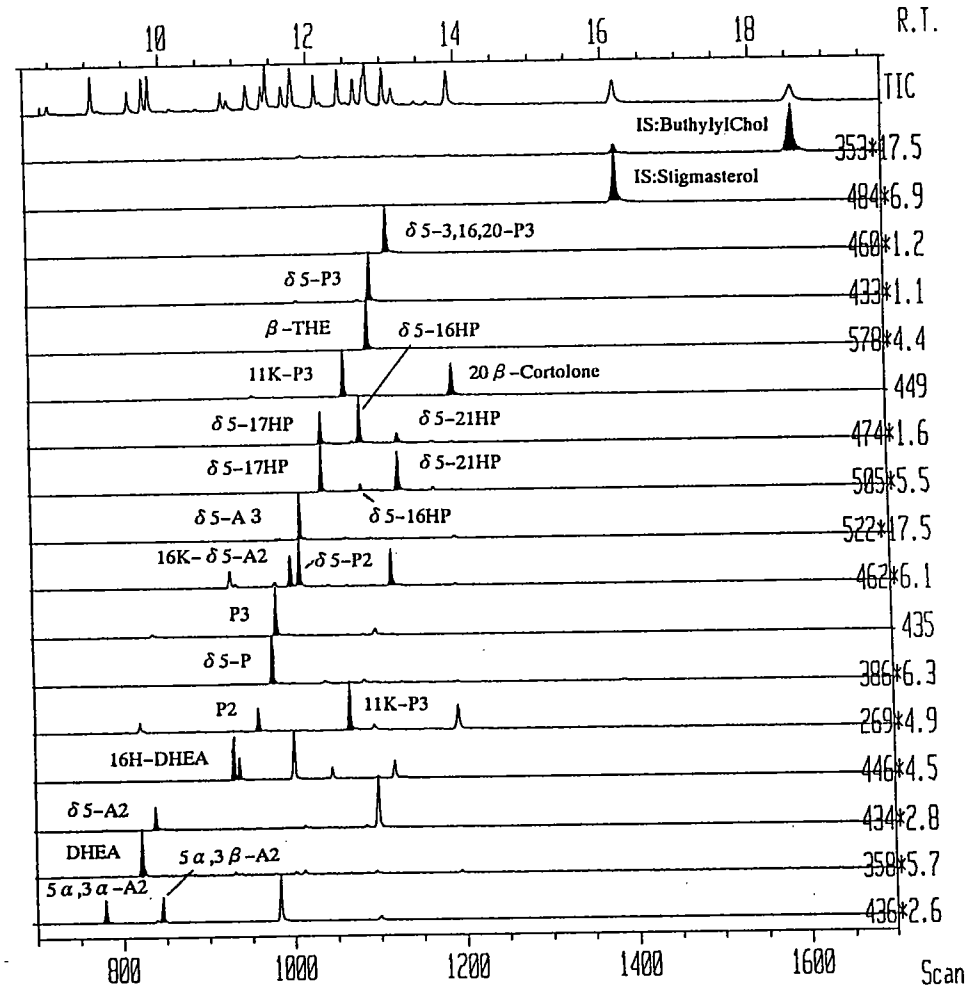
Figure–2–4 EI mass spectra of Methoxyme–TMS steroids.



Figure–2–5 EI mass spectra of Methoxyme–TMS steroids.



Figure–3 Mixture Group–1



Figure–4 Mixture Group–2

Mass chromatogram of the authentic standard mixture of methoxyme–TMS steroids.

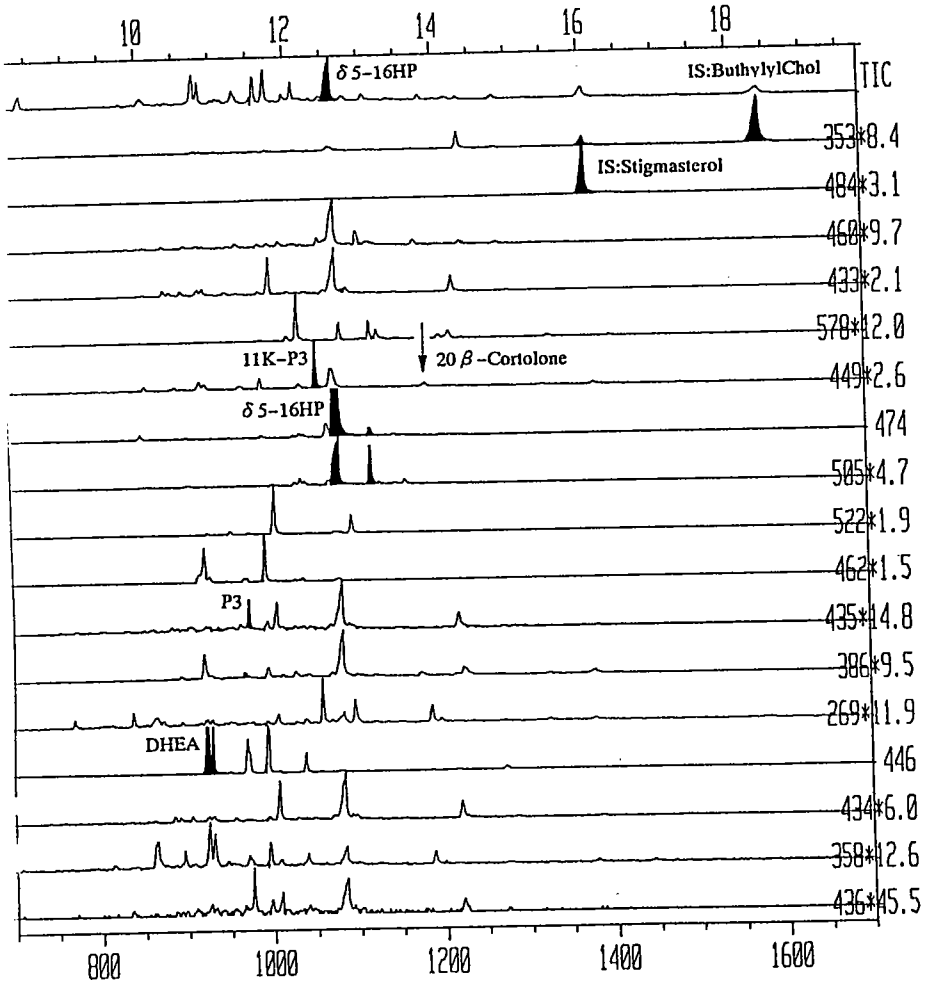


Figure-5 21-Hydroxylase deficiency

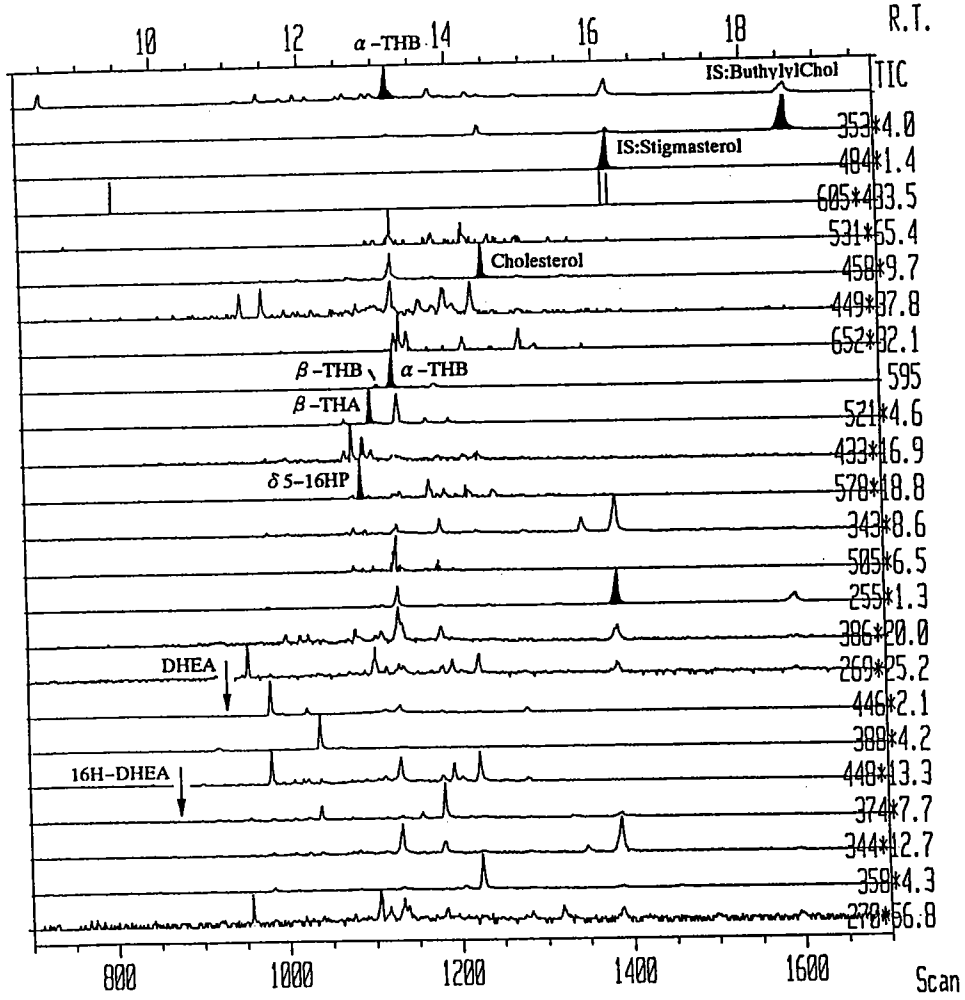
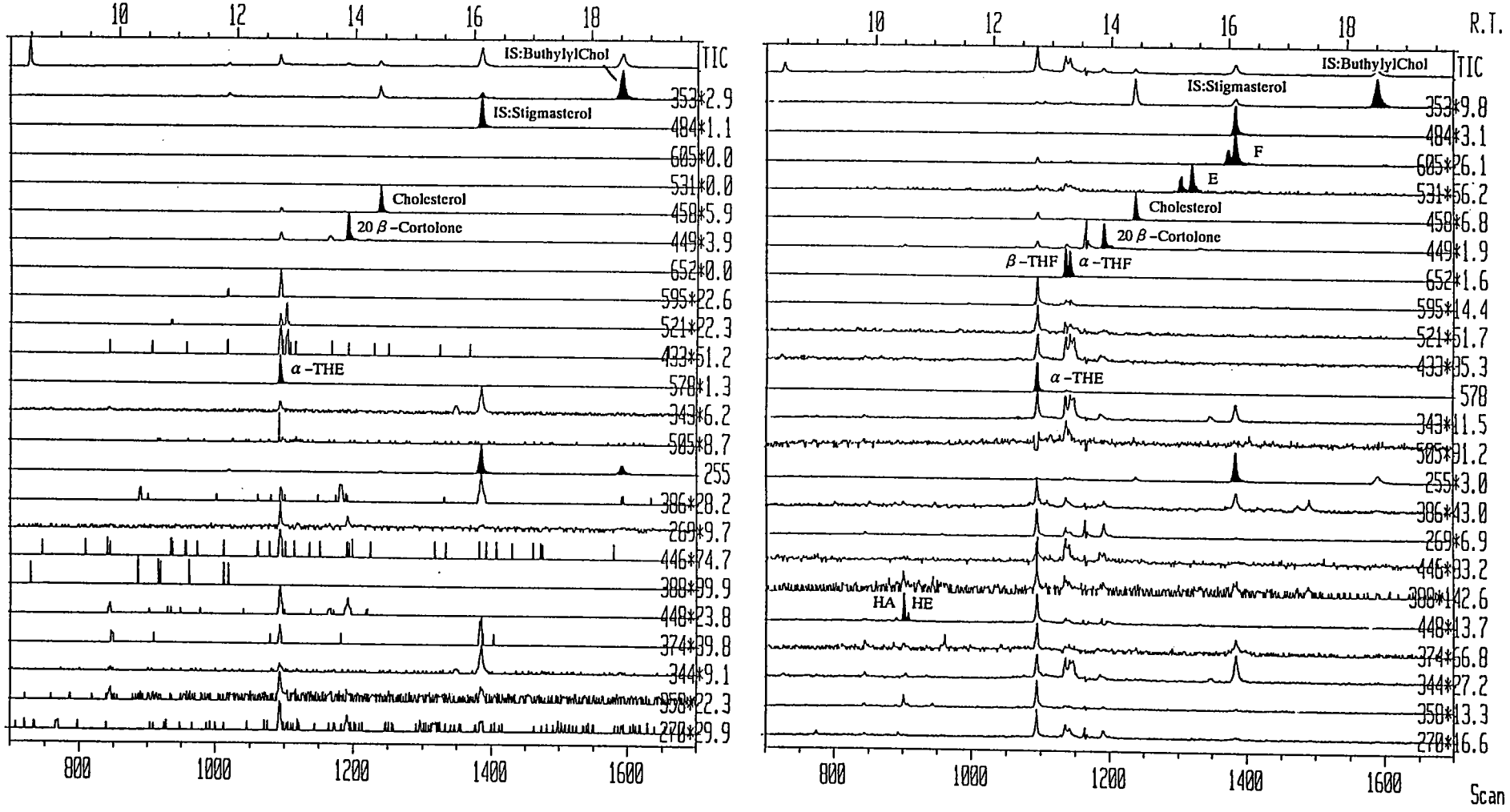


Figure-6 17α-Hydroxylase deficiency

The urinary steroid profiles of the patient with Congenital Adrenocortical Hyperplasia (CAH).



A. Before medical treatment

B. After oral application of Cortisol

Figure-7 The urinary steroid profiles of the infants with P-450 scc deficiency.

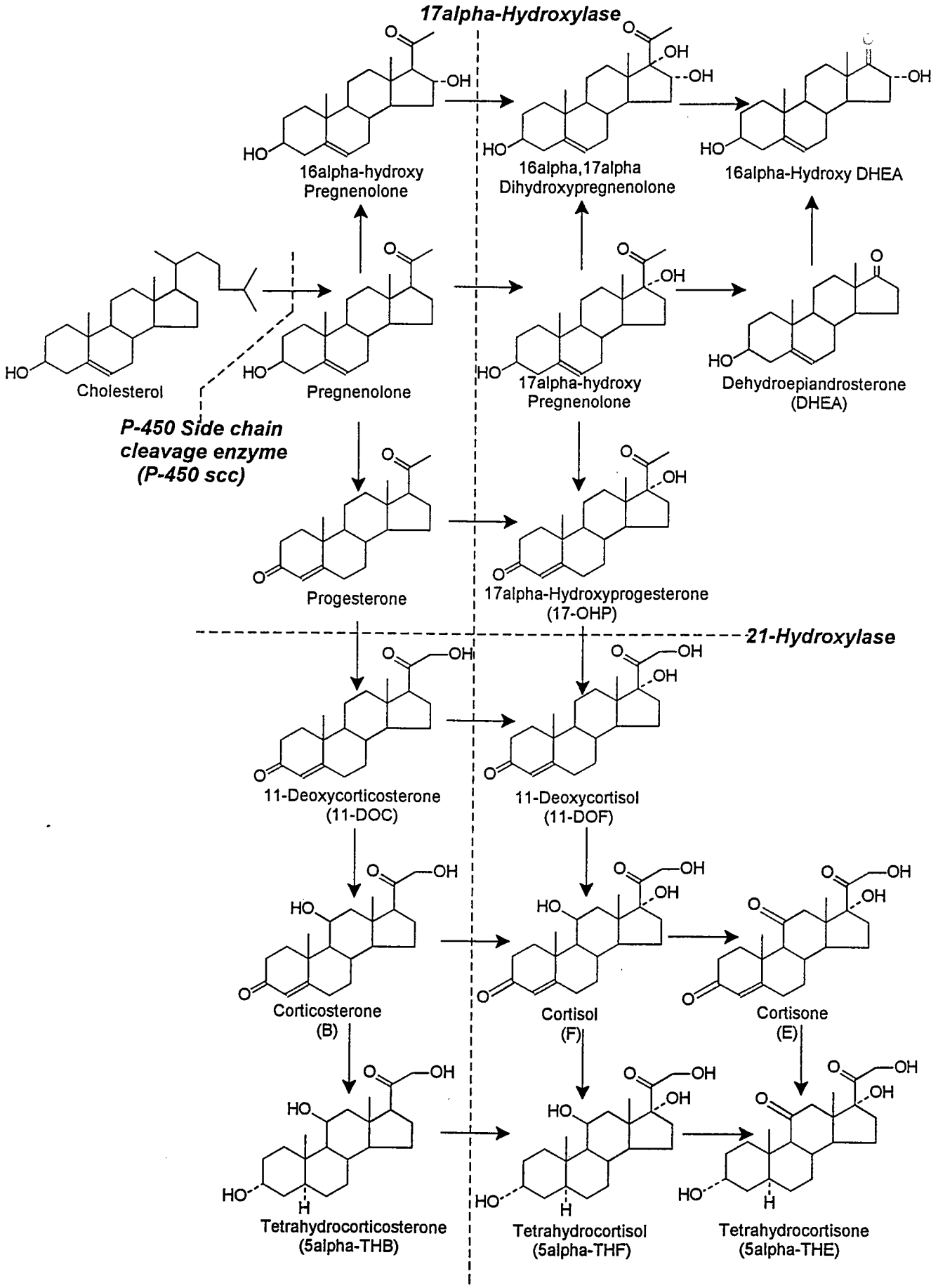


Figure-8 Metabolic pathway of steroids