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SEMI-MICRO QUANTITATIVE ANALYSIS OF COMPLEX URINARY STEROID MIXTURES IN HEALTHY AND DISEASED STATES

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SUMMARY

A method for the determination of urinary steroid hormones in healthy and diseased states is described. The use of a Van den Berg all-glass injector coupled to a fused silica column has shown a dramatic increase in sensitivity and a significant reduction in gas chromatography analytical time. The increase in sensitivity also eliminates the need for processing large volumes of urine.

The method has proven to be rapid, precise, reproducible and sensitive. Also, column life is increased due to the absence of solvent. This technique has shown to have broad applications in the analysis of such classes of compounds as sugars, steroids, prostaglandins and fatty acids.

INTRODUCTION

Multicomponent analysis of urinary steroids relies on the techniques of gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS).

The early work of Sparagna et al. [1], on urinary steroid extracts showed it necessary, in some instances, to separate certain 17-ketosteroids prior to analysis by GC. They showed that dehydroepiandrosterone (DHA) and androsterone co-eluted on packed columns and quantitative data could only be obtained if these compounds were previously separated by some other chemical method. Alternatively, these compounds could be distinguished by analyzing mixtures collected in different fractions from a primary gas chromatograph and reinjecting selected fractions on a second GC column containing a different phase. Although the overall precision was good the analysis was time consuming and also required large volumes (approx. 100—200 ml) of urine to achieve the desired sensitivity.

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Following the introduction of open tubular columns, many workers [2—9] have demonstrated the ability to analyze urinary steroid extracts containing those compounds previously considered difficult to separate using conventional packed column GC. However, the GC analytical time was still considerable (e.g. 90—120 min) and the GC resolution was inadequate for the separation of some of the urinary steroids. Also, because of the low column capacity and the sensitivity of the column coating to the presence of large solvent loads, the majority of the sample had to be split and vented into the atmosphere (split injection). Therefore, in order to obtain comparable sensitivity, highly concentrated samples were required for analysis and this again involved processing large volumes (approx. 25—50 ml) of urine. Today there are a number of GC inlet systems currently in use with capillary GC columns. Of these split, mentioned above, and splitless have gained most popularity.

Splitless injection is strongly dependent on the "solvent effect" [10]. It requires that the initial column temperature be below the boiling point of the solvent utilized in the analysis. This is necessary to achieve a narrow band of the compounds to be analyzed at the head of the column; a prerequisite for good chromatographic resolution. A variation of splitless injection is the on-column method where the injection port is absent and the solvent and analyte are placed in a fine band at the head of the column in a cool oven.

Using split, splitless, and on-column injection methods; solvent traverses the column. During GC analysis this is represented by a solvent front at the beginning of the chromatogram. The presence of solvent in the GC column can cause premature degradation of column performance. Similarly, during GC—MS analysis, repeated exposure of the ion source of the mass spectrometer to solvent can lead to rapid defocusing of the ion beam caused by contamination of the lenses. To avoid this degradation of performance many investigators use complex valving systems to divert the solvent peak.

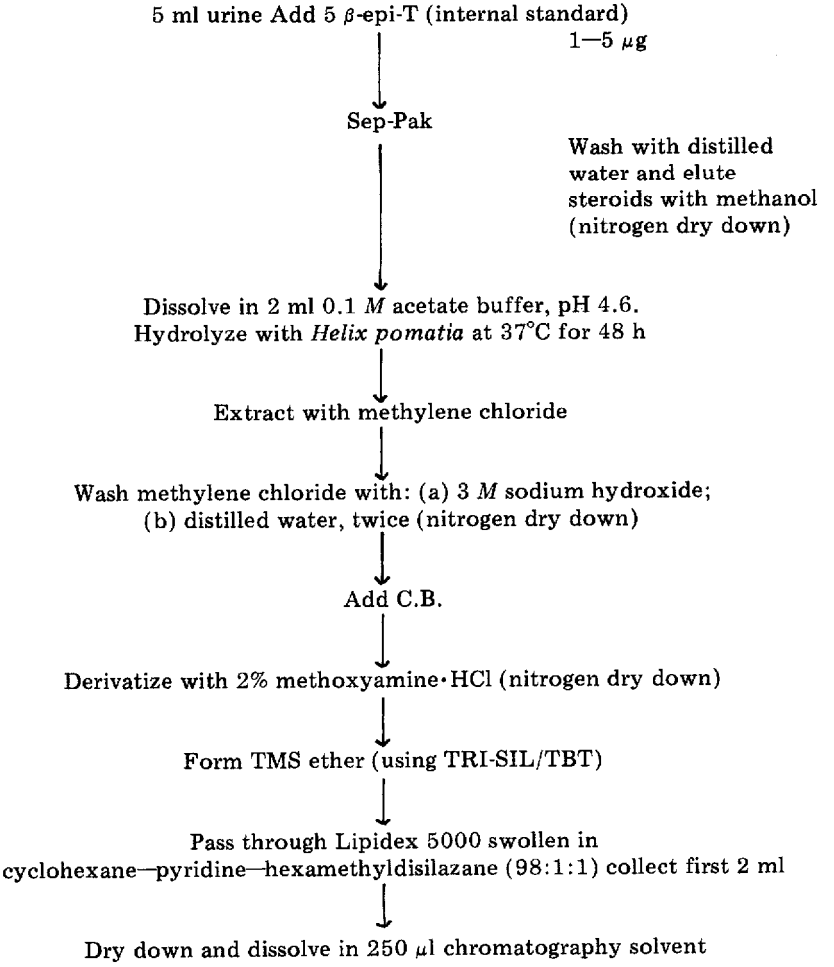
In our laboratory we have used a modified Van den Berg falling needle injector [11, 12]. The modifications include using swagelok connections and needle valves to control helium inlet and outlet gas flows. These allow precise and reproducible gas regulation. Finally, using glass capillary tubing with an inner diameter very close to the outer diameter of the column in the area situated in the injection port decreases dead space and improves GC resolution. This type of inlet system has overcome most of the problems associated with the other forms of inlet systems mentioned above. By eliminating the need to vent the solvent or split the sample there is a dramatic increase in sensitivity, since all the sample is deposited on to the column. It also eliminates the passage of solvent through the column. Because of these two fundamental parameters there is a profound decrease in GC analytical time. Moreover, by using this form of inlet system, relatively small volumes of urine, 1—5 ml, can be assayed with excellent sensitivity and precision.

This technique has shown to be of immense value for the analysis of small volumes of urine. It would also be useful where the compounds of interest are in low concentration or where the total sample volume is small.

EXPERIMENTAL

Twenty-four hour urines were collected and stored at −70°C until the day of analysis. All glassware used throughout the analytical procedure was acid washed and silanized.

Urine was allowed to completely thaw and aliquots of 1—5 ml removed. 5 μg per ml of urine of 5β-dihydro-epitestosterone (Steraloids) were added in ethanol to each as an internal standard for overall recovery. The aliquots were then passed through a Sep-Pak (Waters Assoc.) and a steroid-rich fraction eluted with 8 ml methanol [13]. Solvent was removed under a stream of nitrogen and the steroid conjugates hydrolyzed using the following method. The dried methanolic extracts were dissolved in 2 ml of 0.1 *M* acetate buffer, pH 4.6, followed by 100 μl of *Helix pomatia* digestive juice (Calbiochem-Behring). The mixture was incubated for 48 h at 37°C. Neutral steroids were extracted with 4 ml methylene chloride, this in turn was washed



Scheme 1.

with 1 ml of 3 *M* sodium hydroxide followed by 2 × 2 ml of distilled water. To the organic phase were added 5 μg cholesterol butyrate (CB) as an internal standard for GC. Solvent was removed under a stream of nitrogen and the meth(yl)oxime, trimethylsilyl (TMS) ether derivatives were prepared by the method of Thenot and Horning [14] as follows: 100 μl of 2% methoxyamine·HCl in pyridine were added to the dried extract and allowed to derivatize at 60°C overnight. The pyridine was removed under nitrogen and residual moisture removed in vacuo at 60°C. Trimethylsilyl ethers were prepared by reacting the extract with 100 μl Tri-Sil/TBT reagent [composed of N-(trimethylsilyl)-imidazole (TMSI)—N,O-bis(trimethylsilyl)acetamide (BSA)—trimethylchlorosilane (TMCS) (3:3:2, v/v)] (Applied Science Labs.) for 2 h at 100°C. Excess reagent was removed under a stream of nitrogen and the derivatized extracts suspended in Lipidex 5000 (Packard) slurry swollen in cyclohexane—pyridine—hexamethyldisilazane (98:1:1) [15]. The slurry was added to the top of a 2-cm column of Lipidex 5000 and eluted using the same chromatography solvent. The first 2 ml were collected and evaporated under a stream of nitrogen.

It was found necessary to dilute the derivatized extract equivalent to 5 ml of urine to 250 μl with the chromatography solvent and inject 1 μl.

Gas chromatography

The analysis of urinary steroid extracts was performed on a Hewlett-Packard 3710A gas chromatograph fitted with a modified Van den Berg falling needle injector. This was coupled to a 30 m × 0.25 mm, film thickness 0.25 μm, DB1 fused silica open tubular column (J & W Scientific, Cordova, CA, U.S.A.). The carrier gas was helium, velocity 20 cm/sec and the make-up gas nitrogen with a flow-rate of 20 ml/min. Hydrogen and air flow-rates were 30 ml/min and 300 ml/min, respectively. The injector and flame ionization detector temperatures were 300°C.

GC analysis of urinary steroid extracts was performed with an initial column temperature of 200°C for 4 min, then increased to a final temperature of 300°C at a rate of 4°C/min. The recorder chart speed was 1.25 cm/min.

Gas chromatography—mass spectrometry

Analyses were performed on a Hewlett-Packard 7620A gas chromatograph fitted with a modified Van den Berg injector. The GC column was a 30 m × 0.25 mm, film thickness 0.25 μm, DB1 fused silica column. The carrier gas was helium, velocity 40 cm/sec. Make-up gas was not required since the column was directly inserted into the ion source. The injection block and transfer line temperatures were 300°C.

This was interfaced to a Vacuum Generator (VG) MM-16 low resolution magnetic sector instrument. The multiplier was 1.75 kV, with a gain of $2 \cdot 10^{-6}$. The electron energy was 70 eV, source temperature 200°C and an accelerating voltage of 4 kV. The scan rate was 1 sec per decade. Results were output to a VG 2050 data system.

GC—MS analysis of urinary steroid extracts was performed using the same temperature conditions as stated previously.

RESULTS AND DISCUSSION

Fig. 1 is a typical GC—flame ionization detector trace of a synthetic steroid mixture containing most of those compounds of clinical importance. Each peak is equivalent to 20 ng of steroid. Fig. 2 is a total ion chromatogram of the same mixture obtained from the mass spectrometer data system. Comparison of the two traces indicates almost identical GC resolution. The dif-

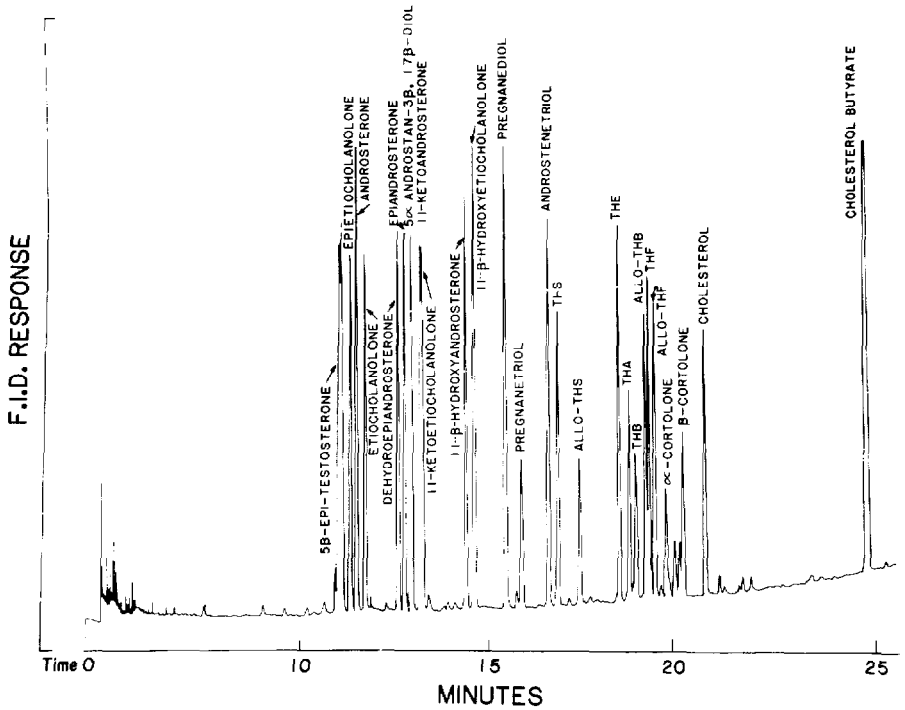


Fig. 1. Gas chromatogram of a synthetic steroid mixture as their meth(yl)oxime TMS ethers. Each peak is equivalent to 20 ng of authentic standard.

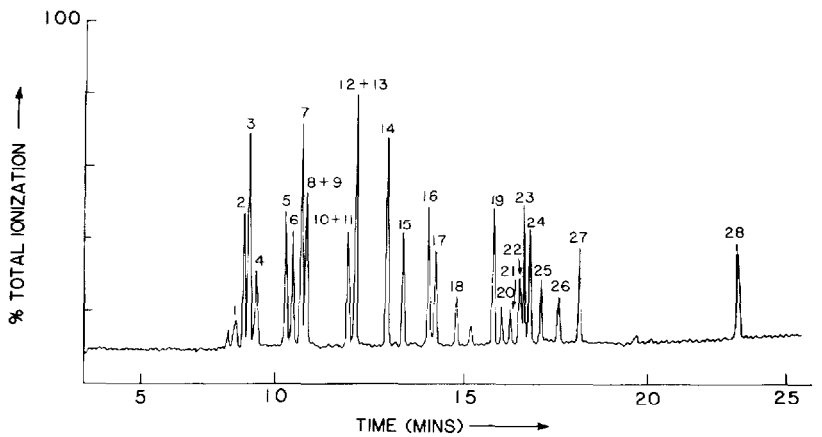


Fig. 2. Total ion current of a synthetic steroid mixture, as their meth(yl)oxime TMS ethers. Each peak is equivalent to 20 ng of authentic standard. See Table I for peak identification.

TABLE I

COMPOUNDS REPORTED IN FIGS. 1—5

Peak No.	Abbreviation	Trivial name	Systematic name	Methylene units
1	5β-dihydro-epi-T	5β-Dihydroepitestosterone	5β-Androstan-17α-ol-3-one	25.31 (25.34)*
2	Epi-E	Epietiocholanolone	5β-Androstan-3β-ol-17-one	25.53
3	A	Androsterone	5α-Androstan-3α-ol-17-one	25.60
4	E	Etiocholanolone	5β-Androstan-3α-ol-17-one	25.71
5	DHA	Dehydroepiandrosterone	Androst-5-en-3β-ol-17-one	26.30
6	Epi-A	Epiandrosterone	5α-Androstan-3β-ol-17-one	26.43
7	And-diol	Androstanediol	5α-Androstane-3β,17β-diol	26.51
8	11 Keto A	11-Ketoandrosterone	5α-Androstan-3α-ol-11,17-dione	26.69
9	11 Keto E	11-Ketoetiocholanolone	5β-Androstan-3α-ol-11,17-dione	26.69
10	17α-OH Preg	17α-Hydroxypregnanolone	5β-Pregnane-3α,17α-diol-20-one	27.35
11	11β-OH-A	11β-Hydroxyandrosterone	5α-Androstane-3α,11β-diol-17-one	27.35
12	Preg	Pregnanolone	5β-Pregnan-3α-ol-20-one	27.51
13	11β-OH-E	11β-hydroxyetiocholanolone	5β-Androstane-3α,11β-diol-17-one	27.51
14	P. diol	Pregnanediol	5β-Pregnane-3α,20α-diol	28.08
15	P. triol	Pregnanetriol	5β-Pregnane-3α,17α,20α-triol	28.31
16	A. triol	Androstenetriol	Androst-5-ene-3β,16α,17β-triol	28.78
17	THS	Tetrahydro substance S	5β-Pregnane-3α,17α,21-triol-20-one	28.89
18	Allo-THS	Allo-tetrahydro substance S	5α-Pregnane-3α,17α,21-triol-20-one	29.28
19	THE	Tetrahydro compound E	5β-Pregnane-3α,17α,21-triol-11,20-dione	29.95
20	THA	Tetrahydro compound A	5β-Pregnane-3α,21-diol-11,20-dione	30.14
21	THB	Tetrahydro compound B	5β-Pregnane-3α,11β,21-triol-20-one	30.27
22	Allo-THB	Allo-tetrahydro compound B	5α-Pregnane-3α,11β,21-triol-20-one	30.46
23	THF	Tetrahydro compound F	5β-Pregnane-3α,11β,17α,21-tetrol-20-one	30.47
24	Allo-THF	Allo-tetrahydro compound F	5α-Pregnane-3α,11β,17α,21-tetrol-20-one	30.56
25	α-Cortolone		5β-Pregnane-3α,17α,20α,21-tetrol-11-one	30.81
26	β-Cortolone		5β-Pregnane-3α,17α,20β,21-tetrol-11-one	31.11
27	Cholesterol		Cholest-5-en-3β-ol	31.56
28	CB	Cholesterol butyrate	Cholest-5-en-3β-ol *n*-butyrate	34.66

*Methylene unit value in parentheses indicates the *syn*- and *anti*-isomers.

ferences observed in their relative intensities are due to the independent means of detection. Table I lists these compounds using the trivial name, systematic name and methylene unit (MU), in order of elution from the GC column.

Quantitation was obtained by analyzing a synthetic steroid mixture of known concentration. The peak areas were integrated by a Hewlett-Packard 3385A system. A response factor (RF) was then computed for each steroid in the mixture relative to the internal standard 5β-dihydro-epitestosterone, using the formula

$$RF = \frac{(\text{Conc. U.K.}) \equiv (\text{Area STD})}{(\text{Conc. STD}) \equiv (\text{Area U.K.})}$$

where: Conc. U.K. = concentration of unknown peak or synthetic standard; Area U.K. = area of unknown peak or synthetic standard concentration; Conc. STD = concentration of internal standard (5β-dihydro-epitestosterone); Area STD = area of internal standard (5β-dihydro-epitestosterone).

These response factors were put into a BASIC program. Using the peak areas and the corresponding internal standard, concentrations were then computed for each steroid in our patient samples. Reproducibility and precision for both concentration and MU were determined on the GC system routinely employed. This was achieved by ten replicate analyses of a normal adult male 24-h urine. A summary of these results is shown in Tables II and III. Also included in Table II is a summary of percentage recoveries of some clinically important steroids. This was performed in model experiments using authentic standards. The values represent the recovery at the final stage of purification.

Except for THA and THB, the overall precision for those steroids indicated is excellent. The discrepancy in quantitating those two compounds may be partly due to their relatively low levels normally excreted in urine. It is there-

TABLE II

TEN REPLICATE DETERMINATIONS OF AN ADULT MALE 24-h URINE

	Mean (mg/24 h)	S.D.	Coefficient of variation (%)	Recovery (%)
Androsterone (A)	4.19	0.058	1.38	54
Etiocholanolone (E)	3.44	0.061	1.77	52
11-keto A + E	0.97	0.07	7.22	63
11β-OH-A	1.63	0.074	4.54	62
11β-OH-E	0.81	0.048	5.87	63
Pregnanetriol	1.08	0.040	3.7	57
THE	1.67	0.134	8.02	68
THA	0.152	0.020	13.16	52
THB	0.33	0.043	13.03	57
Allo-THB	0.50	0.049	9.8	61
THF	0.79	0.063	7.9	64
Allo-THF	0.54	0.039	7.22	65
α-Cortolone	0.24	0.023	9.58	65
β-Cortolone	0.19	0.025	13.16	63
Cholesterol	0.30	0.040	13.33	50

TABLE III

METHYLENE UNIT DETERMINATION ON NINE REPLICATE SAMPLES OF THE STEROID STANDARD USING THE DRY INJECTOR

	Methylene unit reproducibility						
	Andro	Etio	DHA	11β-OH-A	THE	THF	A-THF
$\bar{x}$	25.60	25.71	26.30	26.42	29.95	30.47	30.56
σ_{n-1}	0.007	0.008	0.009	0.006	0.006	0.007	0.007

fore possible that the integrating system employed does not reproducibly quantitate peak areas of this order of magnitude.

The illustration of methylene unit (MU) reproducibility in Table III was calculated for several steroids present in a normal adult male urine. The calculated standard deviation (S.D.) was found to be insignificant for the nine determinations performed. Indeed, the data system employed to identify the MU values for those peaks has been calibrated only once in its several months of use.

The establishment of a normal range from healthy individuals is of prime importance if the evaluation of pathological conditions is to be determined. A number of workers [1, 2, 16] have reported values for their analytical procedures. Since most of their analyses were performed using the more conventional GC inlet systems, it was considered necessary to establish a normal range using the system and techniques we were using.

Twenty-four hour urine was collected from nine healthy, normal adult

TABLE IV

NORMAL ADULT MALE URINARY STEROIDS (mg/24 h)

Steroid	Range 95% population (n=9)	Literature values [8]	Mean [13]
Androsterone	3.09 —6.96	1.69	4.47
Etiocholanolone	3.07 —5.03	2.08	2.99
DHA	0.51 —1.74	0.42	2.17
11-Keto (A and E)	0.16 —1.82	0.53	0.49
11β-OH-A+17α-OH preg.	1.36 —3.84	—*	0.77
11β-OH-E+preg.	0.56 —1.52	—	0.47
Pregnanediol	0.04 —1.51	0.80	—
Pregnanetriol	0.65 —1.90	1.64	—
Androstenetriol	2.05 —5.06	—	—
THE	1.13 —3.58	6.06	—
THA	0.72 —1.40	—	—
THB	0.006—0.45	—	—
Allo-THB	0.11 —0.84	—	—
THF	0.60 —1.33	—	—
Allo-THF	0.57 —1.35	—	—
α-Cortolone	0.21 —0.45	—	—
β-Cortolone	0.19 —0.43	—	—
Cholesterol	0.12 —1.16	—	—

*Dash (—) indicates these compounds were not quantitated.

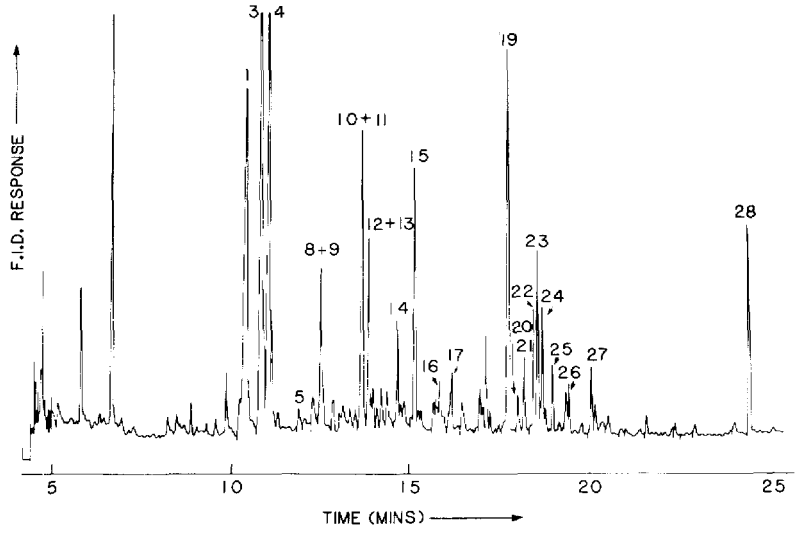


Fig. 3. Gas chromatogram of a normal adult male 24-h urinary extract, as the meth(yl)oxime TMS ethers. The extract is 1/250 of a 5-ml aliquot of urine. See Table I for peak identification.

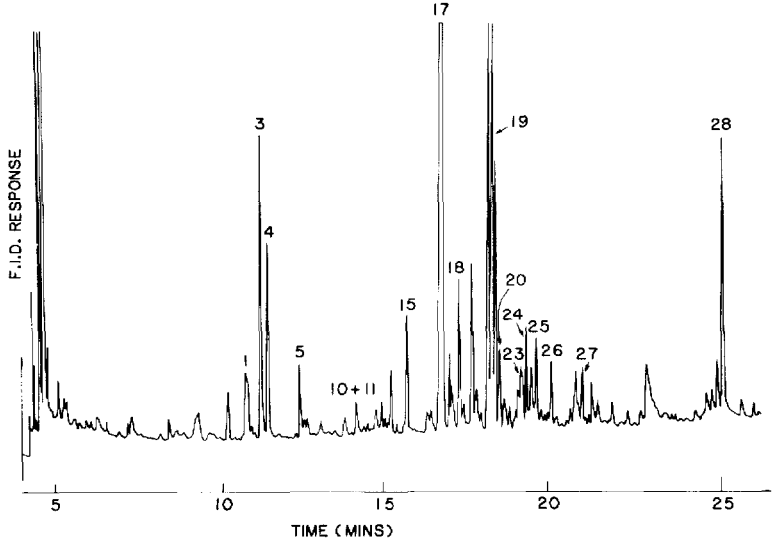


Fig. 4. Metabolic profile of a urinary extract from a patient treated with metyrapone for Cushings syndrome. See Table I for peak identification.

male volunteers working in the laboratory. Their ages ranged between 25 and 37 years, with a mean age of 31 years. Aliquots of 5 ml were removed and analyzed as described previously. Results were expressed as mg per 24 h and are summarized in Table IV. The range was calculated as ±2 S.D. of the normal mean for each steroid.

Comparison of these data with results reported by other investigators can often be misleading due to differences in methodology. Many authors include hot acid hydrolysis in their procedure. However, because of both the similarity

of our data to those of other investigators and the precision and reproducibility, we feel that the data reported in this paper indicate that the acid hydrolysis step may not be necessary.

Fig. 3 is an example of a GC metabolic profile from a normal adult male. It is dominated by the major urinary androgens, androsterone and etiocholanolone, peaks 3 and 4, respectively. Cortisol metabolites at this period of life are greatly diminished. Comparison of this profile with one from a patient being treated with metyrapone, Fig. 4, for Cushings syndrome, shows an elevated level of THS, peak 17. In this instance, THS has been further metabolized to its corresponding 20α- and -β-pregnanetetrols. These are the un-numbered peaks eluting either side of THE (peak 19). Metyrapone acts by inhibiting the enzyme 11β-hydroxylase, which is responsible for the conversion of 11-deoxycortisol (substance S) to cortisol (compound F). Consequently, excessive amounts of 11-deoxycortisol are built up in plasma and are excreted in the urine as tetrahydro substance S. Another example of disease profiling using this technique is shown in Fig. 5. The urinary steroid profile is that from a patient suffering from adrenogenital syndrome. In this condition the enzyme responsible for 21-hydroxylation is genetically absent. Biochemically, the pathway to the formation of cortisol is blocked at the conversion of 17α-hydroxyprogesterone to substance S due to the absence of the 21-hydroxylase enzyme; 17α-hydroxyprogesterone in turn is excreted in considerable amounts as its urinary metabolite of pregnanetriol, peak 15.

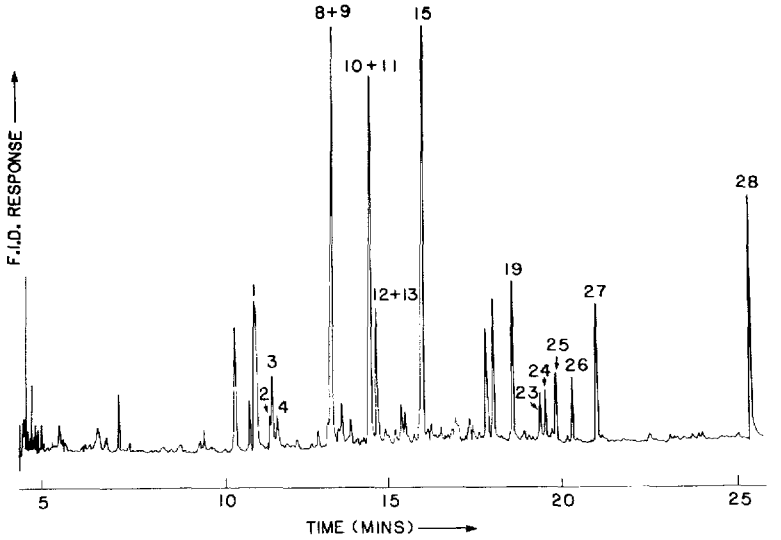


Fig. 5. Metabolic profile of a urinary extract from a patient with 21-hydroxylase deficiency. Patient was being treated with prednisone. See Table I for peak identification.

CONCLUSION

Routine analysis of urinary metabolic profiles is still hindered by the lengthy enzymatic hydrolytic procedure. The method described in this paper still incorporates this stage. However, incorporating fused silica capillary columns coupled to a Van den Berg injector has greatly reduced the GC analysis time,

with little or no sacrifice in GC resolution. Because of the excellent resolution and sensitivity achieved with the use of these columns it is possible to analyze significantly reduced volumes of urine. This has eliminated the cumbersome extraction procedures with large volumes of toxic solvents that are necessary when large volumes of urine are used and has made it possible to rapidly prepare larger numbers of samples prior to hydrolysis.

The stability and reproducibility of this method is exemplified in the Results section. Standard deviations for MU reproducibility were shown to be very small. Indeed, even after several months of use the agreement of these values to their original are still excellent.

The precision of this method is quite remarkable considering the small volumes of urine analyzed. The majority of the analytes having a coefficient of variation of less than 10%, and most of the major metabolites less than 5%.

The normal ranges presented in Table IV are in very good agreement with those methods that incorporate hot acid solvolysis [1, 13]. This could be indicative of the efficiency of the enzyme system employed in this method, since the mixture does contain both the arylsulfatase and β-glucuronidase. An alternative explanation would be that the enzyme cleaves the conjugates reproducibly but incompletely. However, studies where we have extended the incubation time to 75 and 96 h have not changed the reproducibility obtained with 48-h incubations. Furthermore, preliminary results using radiolabeled steroid sulfate conjugates has indicated that the enzymatic hydrolysis is more than 95% complete. However, further investigations into this area are still in progress to confirm this aspect.

The flexibility of fused silica columns has enabled the column to be directly coupled to the mass spectrometer. Also the absence of solvent has circumvented the need for the conventional type of separator usually incorporated into the system. Using this system aliquots previously analyzed by GC can now be examined by GC—MS without further manipulation of the derivatized extract, for example eliminating the need to further concentrate the sample or change solvent systems.

ACKNOWLEDGEMENTS

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