Evaluation of Hydrolytic Products of 17-Ketosteroids by Means of Gas-Liquid Chromatography

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In the course of evaluating urinary 17-ketosteroid (17-KS) gas-liquid chromatographic patterns, a number of peaks with retention times shorter than that of 17-KS were constantly observed. This background material was found to be degradation products of 17-KS produced by HCl hydrolysis. A study with pure 17-KS as a mixture and as individual steroids was used to identify the steroids responsible for most of the 9 compounds with retention times less than etiocholanolone. Hydrolysis in ethyl acetate containing perchloric acid produced smaller amounts of degradation products than HCl-hydrolysis. Enzymatic hydrolysis appears to be the most acceptable system for preparation of the extracts from urine for this system of assay.

The hydrolysis of conjugated urinary 17-ketosteroids (17-KS) by boiling or heating with mineral acids results in the formation of degradation products (1-9). In this regard, the most labile and thoroughly studied 17-KS is dehydroepiandrosterone (DHA) (1-3, 6-9). Degradation of steroids by acid hydrolysis does not cause serious decrease in the total amount of 17-KS when measured by the Zimmerman reaction (6) and does not assume importance unless an individual 17-KS is to be identified. It is presumed that most of the degradation products react with the Zimmerman reagent. However, Sheath (7) was able to detect losses of DHA and androsterone when these steroids were added to urine, and the specimen then subjected to acid hydrolysis.

Evidence exists showing that the glucuronoside-conjugated 17-KS is adequately hydrolyzed by enzymatic systems (10), although the problem of inhibitors in urine remains unsolved. The use of sulfatases for splitting steroids conjugated with sulfate is considered unsatisfactory, since inhibitors greatly reduce the effectiveness of this enzyme. For hydrolysis of sulfates, Lieberman et al. (9) introduced the method of solvolysis.

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which was reported to release the steroid from its conjugate without production of artifacts. Burstein et al. (2) in 1960 described the hydrolysis of steroid glucuronosides in ethyl acetate or tetrahydrofurane (THF) in the presence of perchloric acid. The advantage of this method is that the 17-KS conjugates, either glucuronosides or sulfates, can be hydrolyzed by a single process without alteration of the steroid molecules. Recently DePaoli et al. (5) reported a study evaluating the Burstein hydrolytic method for conjugated 17-KS by gas-liquid chromatography. They used ethyl acetate as the organic medium. Both of these investigations indicated that there was little, if any, degradation of the 17-KS during the process of hydrolysis. In 1962, Jacobsohn and Lieberman (6) used THF as organic medium with perchloric acid for hydrolysis since Bernstein reported the formation of acetate derivatives of steroids during the hydrolytic procedure in ethyl acetate. These authors showed that mineral acid treatment of urinary 17-KS conjugates caused disarrangement of many steroids, particularly DHA, 11-ketoetiocholanolone (11-KE), and 11-hydroxyetiocholanolone (11-OHE). They detected that these 11-hydroxylated 17-KS were converted to their Δ⁶ analogues, and by paper chromatography, found them to be mixed with etiocholanolone (E) and androsterone (A). Little derangement of steroidal pattern was noted with the THF medium containing perchloric acid.

Our experience with gas-liquid chromatography convinced us that a relatively large number of degradation artifacts were being formed during acid hydrolysis of urinary steroids and that gas-liquid chromatography was well suited to illustrate the degree of disarrangement. Artifacts were observed following HCl hydrolysis but not after β-glucuronidase hydrolysis (Fig. 1). The artifact patterns produced were somewhat specific to the method of hydrolysis. Many of the products formed appeared to be smaller molecular components, since on SE-30 columns they had retention times shorter than that of E. The effects on 17-KS of hydrolysis by two methods—HCl hydrolysis and perchloric acid hydrolysis in ethyl acetate—was studied by gas-liquid chromatography. The study includes effects on individual 17-KS's and upon a synthetic mixture of 17-KS's made up to resemble the chromatographic pattern of urinary 17-ketosteroids.

**Material and Methods**

**Synthetic Mixture of 17-KS**

The standard mixture was made up in dichloromethane to contain 1.5 mg./ml. of each of the following: A, E, DHA, 11-KE, 11-OHE, 11-hydroxyandrosterone (11-OHA), and 11-ketoandrosterone (11-KA). One
hundred microliters of the mixture was equivalent to 150 μg. of each steroid. Cholestane was added after hydrolysis as an internal standard.

**Individual Standards**

The individual standards of each of the 17-KS listed above were made by adding 100 μg. of each to separate test tubes.

**HCl Hydrolysis**

The standard mixture and individual standards were treated identically to the point where they were reconstituted with dichloromethane containing cholestane. To each tube was added 2 ml. of glacial acetic acid, 8 ml. of water, and 3 ml. of concentrated HCl. The tubes were boiled in a water bath for 10 min., and cooled with running tap water. Each was extracted with 10 ml. of dichloroethane by shaking for 15 min. in a shaker. The aqueous layer was removed by aspiration, and the organic solvent layer shaken with 25-30 NaOH pellets for 15 min. The tubes were centrifuged, the contents filtered, and a 5-ml. aliquot removed and brought to dryness under a stream of nitrogen at 55°.

**Perchloric Acid Hydrolysis**

To the dried residue of the synthetic mixture and individual standards, was added 15 ml. of a solution containing 0.3 ml. of 70% perchloric acid in 300 ml. of dry ethyl acetate. The contents of the flask were transferred to 50-ml. centrifuge tubes, and 5 ml. of 10% (w/w) KOH was added. The tubes were shaken well and the ethyl acetate evaporated under a stream of nitrogen at 55°. The brown layer was extracted twice with 50 ml. of a mixture of hexane and benzene (1:1). The extract was filtered through Na₂SO₄, and evaporated to dryness under a stream of nitrogen at 55°.

**Hydrolysis, β Glucuronidase**

To a test tube containing 10 ml. of water, 1 ml. of 1 M acetate buffer, pH 5, and 3 ml. of ketodase (15,000 U.) were added. Individual standards in their organic solvents (equivalent to 100 μg.) were added to this mixture, and 100 μl. of the standard mixture was added to a separate series. The tubes were then placed in 37° water baths for 72 hr., during which time the organic solvent evaporated. The residues were extracted 3 times with dichloroethane. The extracts were washed twice with 10 ml. of 0.1 N NaOH and twice with 10 ml. of water. An aliquot equivalent to 80% of the original extracting volume was removed. This was brought to dryness under a stream of nitrogen at 55°.

**Internal Standard**

The dry residue was dissolved in 50 μl. of dichloromethane containing approximately 0.3 μg. of cholestane/μl. (range 0.1-0.5 μg./μl.).
Measurement

A Barber-Colman (Model 15*) gas-liquid chromatographic instrument with an argon ionization detector was used. A W-shaped glass column, with a total length of 12 ft., and an internal diameter of 1/8 in. (3-4 mm.) was packed with 1% (w/w) silicone gum (SE-30), liquid phase, on solid support Gas Chrome-P 100-140 mesh.† The column was conditioned initially at 250° for 24 hr. in an atmosphere of argon and was subsequently operated at 235°. The detector was operated at 270°, and the flash heater at 285°. The voltage in the detector was set at 600. Argon pressure was 60 p.s.i. Samples were injected with a 10μl syringe (Hamilton, No. 701-N†).

Results and Discussion

Figure 1 shows gas-liquid chromatography patterns of 17-KS from normal urine hydrolyzed by two methods (HCl and β-glucuronidase) compared to nonhydrolyzed 17-KS standards. Arbitrarily, the peaks with shorter retention time than E were numbered from 1 to 9 commencing with the peak having the longest retention time. As indicated in Fig. 1, about 9 peaks have been found in chromatograms of hydrolyzed urinary 17-KS and their respective retention times were found to be consistent. In addition to the production of pre-E peaks, a number of other changes were noted with HCl hydrolysis such as distortion and decrease of 17-KS peaks, production of peaks with retention times identical to one or more of the 17-KS's, and occasional peaks with retention time differing from 17-KS. No attempt was made to identify these new compounds produced by HCl hydrolysis. Those peaks having retention times similar to one of the 17-KS's were given the same designation with a prime notation, e.g., 11-KA'. Compounds represented by peaks unrelated to known 17-KS were designated X', X'', etc., with increasing retention time. It is apparent that a number of 17-KS's undergo degradation during HCl hydrolysis and contribute to the pre-E peaks, new peaks, and peaks with the same retention time as a 17-KS. Figures 2 and 3 are summaries of the results of HCl and HClO₄ hydrolysis showing interrelationship between breakdown peaks and specific 17-KS standards. The chromatographic pattern of the standard mixture after HCl hydrolysis was different from that of the nonhydrolyzed mixture (Fig. 2) and resembled those seen in urine samples hydrolyzed with HCl (Fig. 1). Similar patterns were observed for HClO₄ hydrolyzed standards and urine.

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Examination of the effects of HCl hydrolysis of individual 17-KS standards (Fig. 2 and 3) elicits a number of observations. A small quantity of E is converted to a compound with a retention time equivalent to 11-KA'. A small percentage of A is converted to compounds with reten-

Fig. 1. Diagrammatic representation of gas-liquid chromatograms of nonhydrolyzed standard 17-KS mixture and urinary 17-KS after HCl hydrolysis and β-glucuronidase hydrolysis. In chromatogram showing effects of HCl hydrolysis (center), 9 peaks are shown in the zone preceding etiocholanolone (E) peak. Chromatogram at bottom (enzyme hydrolysis), shows complete absence of pre-E peaks and little, if any, destruction of 11-hydroxylated androsterones or etiocholanolones.
tion times equivalent to 11-KA' and Peak 5. DHA is almost totally de-
composed. A major share is converted to Peak 4 and a small amount
converted to Peaks 7 and X''. A very small quantity of 11-KA is changed
into 11-OHA' and Peak 3, and 11-OHA is almost totally destroyed and
converted to E' with formation of Peaks 2, 6, 7, X' and 11-KE' in small
amounts. A small amount of 11-KE becomes 11-OHE'. Peak 1 is pro-
duced by destruction of 11-OHE. E, A, 11-KA, and 11-KE are only

Fig. 2. Effects of hydrolysis by HCl and perchloric acid (HClO₄) on chromatographic pat-
tern of standard 17-KS mixture and individual standards.
moderately affected by HCl hydrolysis, and approximately 5–10% undergoes conversion to other compounds. On the other hand, 11-OHA, 11-OHE, and DHA are extremely sensitive and approximately 70–95% is converted to other compounds during prolonged hydrolysis. Figures 4 and 5 show that increasing time (10, 20 and 30 min.) of HCl hydrolysis produces greater loss of parent 17-KS, and greater distortion of the pattern of the standard mixture, such that the pattern after 20–30 min. looks very much like the urinary 17-KS chromatogram (Fig. 1) following preliminary hydrolysis with HCl. Urinary steroid chromatograms

![Diagram of 17-KS hydrolytic products](image)

Fig. 3. Effects of HCl and HClO₄ hydrolysis on chromatographic pattern of 11-substituted 17-KS standards.
(Fig. 1) have a number of pre-E peaks, all of which correspond with the peaks produced by HCl hydrolysis of the pure 17-KS standards (Fig. 2 and 3) except for Peaks 8 and 9. Peak 9 develops after NaOH washing and can be shown in pure solvent treated with NaOH. Peak 8 has not been identified. The chromatographic pattern of liver or bacterial β-glucuronidase hydrolyzed standards shows no significant change from the standard 17-KS pattern (Fig. 6). In this figure, the apparent increase in the A-DHA peak is due to the fact that DHA was not included in the standard mixture that was not hydrolyzed. The deletion was made to show a clear relationship of E and A-DHA peaks in the standard steroid mixture.

As mentioned above, hydrolysis in organic media with perchloric acid has been acclaimed as a gentle method which simultaneously hydrolyzes both glucuronoside and sulphate conjugates. In an attempt to evaluate the effect of this method on standards, the same standard mixtures and

![Fig. 4. Chromatographic pattern of standard 17-KS mixture after 10, 20, and 30 min. of HCl hydrolysis. Note progressive increase in Peaks 1 and 4 and decrease in peak for A-DHA. 11-OHE and 11-OHA have undergone degradation.](image)

![Fig. 5. Chromatographic pattern of dehydroepiandrosterone after 10, 20, and 30 min. of HCl hydrolysis.](image)

![Fig. 6. Effects of enzyme hydrolysis (liver and bacterial β-glucuronidases) on chromatographic pattern of standard 17-KS mixture. Apparent relative increase in A-DHA peak is due to fact that DHA was not added to nonhydrolyzed standard mixture.](image)
individual standards of 17-KS were subjected to hydrolysis in ethyl acetate containing perchloric acid (Fig. 2 and 3). The results indicate that this technic is far less injurious to the steroids than is HCl hydrolysis. The method does not produce pre-E breakdown peaks to any great extent. In each study a reagent peak (R) found in the pre-E zone was shown to be a property of perchloric acid. A number of peaks with retention times equal to those of standard 17-KS as well as a number of peaks with retention time different from those of standard 17-KS were found following hydrolysis. This latter group probably represents acetate derivatives which were formed during hydrolysis in ethyl acetate.

A screening procedure based on a 10-min. HCl hydrolysis and gas-liquid chromatography can be used to obtain qualitative information on urinary 17-KS. Perusal of the resulting chromatogram often is sufficient to indicate whether other studies are necessary. We have been able, by comparing the gas-liquid chromatographic patterns of HCl-hydrolyzed and β-glucuronidase hydrolyzed urine samples, to verify that the 10-min. HCl hydrolysis is in itself sufficient to detect pathologic conditions. With the 10-min. HCl hydrolysis, we were able to detect steroid alterations in 3 cases of Cushing’s syndrome caused by ACTH-producing bronchogenic carcinoma that induced marked increase in excretion of 11-OHE and little increase in A or DHA (11, 12), and in a case of adrenal carcinoma associated with polycythemia with increased excretion of 11-OHA and DHA (13).

The biggest drawback to satisfactory application of gas-liquid chromatography to the analysis of urinary steroids is the method of hydrolysis. Although enzymatic technics are the most gentle, they are slow and costly. Sparagna et al. (14) followed β-glucuronidase hydrolysis by solvolysis at low pH in ether for 3 days to insure complete hydrolysis of sulfate conjugates. An enzyme preparation (Glusulase) from the snail *Helix pomatia*, which contains both glucuronidase and sulfatase in high concentration, has been found to be satisfactory for hydrolysis (10, 15). Another limitation of gas liquid chromatography in 17-KS analysis is pyrolysis of those corticoids with dihydroxyacetone side chains into corresponding 17-KS (16), thus giving falsely elevated 17-KS values. Sparagna et al. (14) removed the corticoids in the urinary extract before 17-KS analysis by applying the Girard procedure and then partitioning the extract between methanol and benzene. Complete separation of A and DHA has been achieved by using the trimethylsilyl-ether derivatives (15, 17). Creech (18), using trimethylsilyl ethers of 17-KS and a 2% NGS column, has shown that all 7 of the 17-KS can be separated in a single run. Vestergaard and Claussen (19) have developed a method of hydrolysis of 17-KS which does not cause steroid artifacts. Their method
involves reflux extraction of urine with benzene and relatively concentrated H$_2$SO$_4$. Creech et al. (20) confirmed by gas-liquid chromatography on 2% NGS that the Vestergaard and Claussen method does not produce artifacts of 17-KS and appears to be an acceptable method for hydrolysis of urinary steroids. The method is faster than enzyme techics, but the two methods have not been compared.

References