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Direct Measurement of Testosterone and Epitestosterone Glucuronates and Sulfates with HPLC/MS and HPLC/MS/MS

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INTRODUCTION

The excretion of steroids in urine as the glucuronate and sulfate conjugates presents significant problems for analysis. In general, the conjugtes must be cleaved either enzymatically or by solvolysis. Hydrolysis with β-glucuronidase assumes equivalent (or complete) cleavage of the glycosidic bond, regardless of the structure of the aglycone. In general, enzymatic hydrolysis and solvolysis must be carried out in parallel, and results inferred from the differences between the two results. A direct analysis of the steroid conjugates would have numerous advantages. We report here our intial results with direct analysis of the sulfate and glucuronate conjugates using electrospray HPLC/MS.

EXPERIMENTAL

Synthesis of d_3-testosterone was accomplished starting with androstenedione as described in Figure 1 [1]. Base-catalayzed deuterium exchange resulted in d_7-androstenedione, which was then reduced with $NaBD_4$ to yield d_9-testosterone. Acid-catalyzed proton exchange removed the deuterium from the A ring, resulting in 16,16,17-d_3-testosterone with an overall yield of 50%. d_3-Epitestosterone was synthesized from d_3-testosterone using the Mitsunobu reaction. The nitrobenzoyl ester of d_3-testosterone was cleaved under basic conditions, resulting in 70% yield.

Epitestosterone glucuronate was synthesized in our laboratory using a modification of the Koenigs-Knörr reaction. The yield using the modified reaction was greater than 60%. Synthesis of d_3-testosterone glucuronide was carried out in the same manner, with a yield of 90%. Preparation of d_3-epitestosterone sulfate was accomplished by reaction with triethylamine-sulfur trioxide complex in pyridine. After conversion to the ammonium salt, reaction yield was 75%.

A PE-Sciex API-III triple quadrupole mass spectrometer (Norwalk, CT) equipped with an IonSpray™ interface was used for detection of steroid conjugates. A Beckman model 126 programmable solvent module delivered a gradient of 0.1% glacial acetic acid and 10 mmol/l

ammonium acetate (A solvent) and methanol to which 0.1 % (v/v) glacial acetic acid and 10 mmol/l ammonium acetate had been added (B solvent). Samples were injected with a Rheodyne 7935 injection valve with a 20 μl loop housed in a DuPont forced air oven. HPLC separations were performed on a 1 x 150 mm column packed with 3 μm Hypersil C-18 BDS stationary phase (Keystone Scientific Inc., Bellefonte, PA). Samples were prepared using a solid phase extraction method. [2]

RESULTS AND DISCUSSION

A synthetic scheme for synthesis of epitestosterone glucuronide and sulfate, and or deuterated internal standards for all four steroid conjugates was developed in order to directly measure their concentration in urine. Dehenin has suggested that variations in sulfation and glucuronidation of epitestosterone may account for "naturally elevated" T/E ratios [3]. This method was developed to collect epidemiological data to either support or refute this claim.

The separation of the testosterone and epitestosterone glucuronide and sulfate standards is shown in Figure 2. Only d_3-epitestosterone sulfate is shown in this figure as the internal standard, although we have prepared the other deuterated compounds. Note the diminished response obtained from epitestosterone glucuronide (m/z 465, t_r 7.95 min.). The reason for this low response is under further investigation, but most likely is due to the lability of the glycosidic bond.

A linear calibration curve was observed over the range of 10 – 1000 nmole/L for all four of the steroid conjugates using SIM HPLC/MS. The expected concentrations would be about 200 nmol/L for testosterone glucuronide and 50 nmol/L for epitestosterone glucuronide. Thus, the method has sufficient sensitivity to analyze these components in urine.

Preliminary results on 15 urine samples collected from normal volunteers showed some variation in the relative amount of conjugates. Three individuals had unusually high relative concentrations of testosterone sulfate. Two individuals had high relative concentrations of epitestosterone sulfate.

CONCLUSIONS

An improved synthetic scheme for [16,16,17-$^2H_3$]-ttestosterone aned -epitestosterone has been achieved. By starting with androstenedione, there is no detectable d_0 contamination of the deuterated product. High yields of the epimer were achieved using the Mitsunobu reaction.

The HPLC/MS method has sufficient sensitivity to directly analyze steroid conjugates. It will be important to use IUPAC molar concentrations in order to interpret results, since the masses of testosreone and its conjugates are different. For reference purposes, 100 ng/mL of testosterone is equivalent to 350 nmole/L. Positive ion MS and MS/MS can provide some ion fragments that could be used for structural validation of the steroid.

The stability of the glycosidic bond, especially in the epitestosterone, has been problematic. The cause of the instability is under investigation.

A preliminary study of 15 urine samples suggests that significant variability of the relative amount of the conjugates is observed between individuals. Dehenin's recommendation that epitestosterone sulfate be added to the denominator of the T/E ratio appears too simplistic.

References

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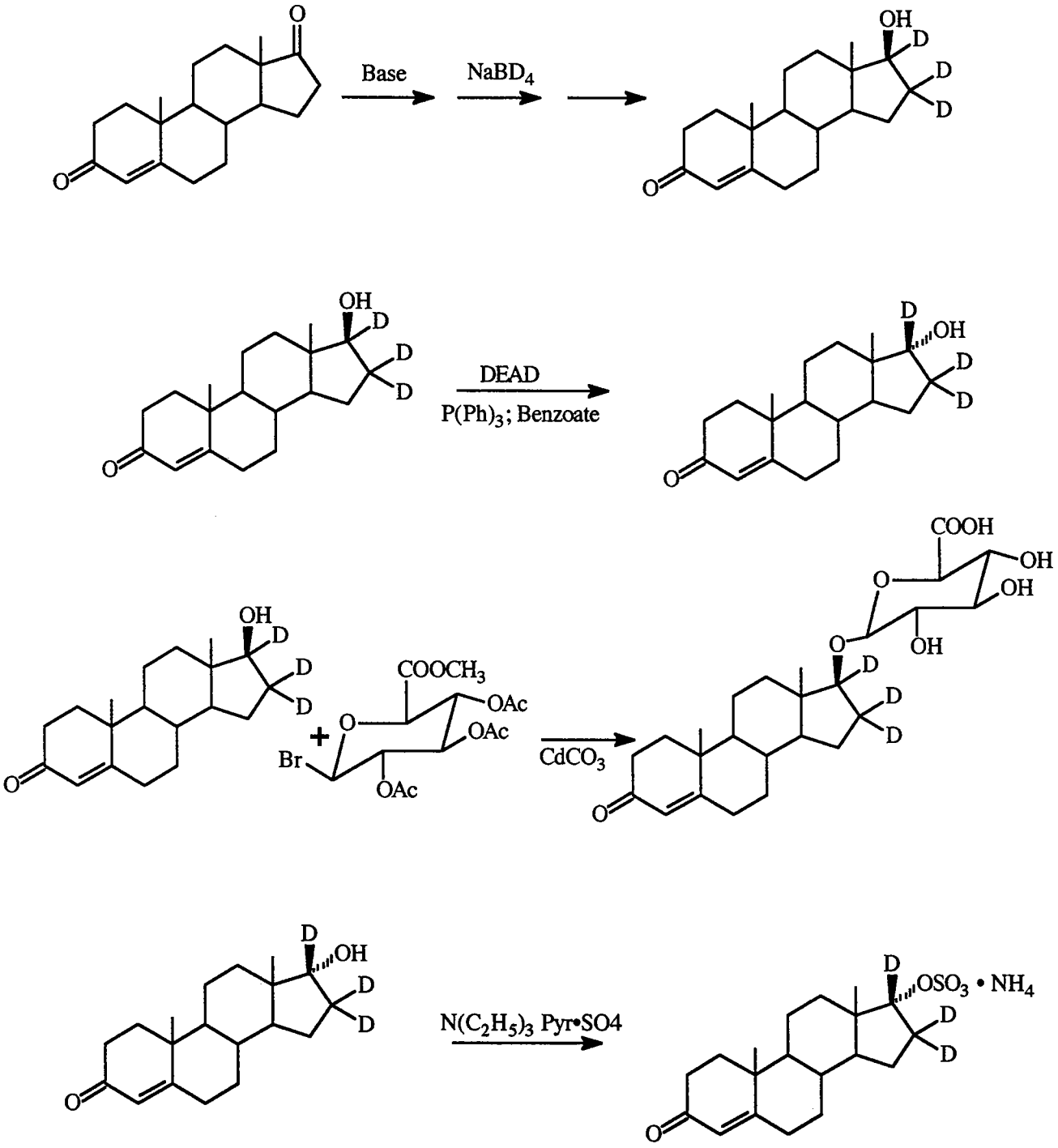

Figure 1. Synthetic scheme for the synthesis of testosterone and epitestosterone conjugates. For specific details, see reference [1].

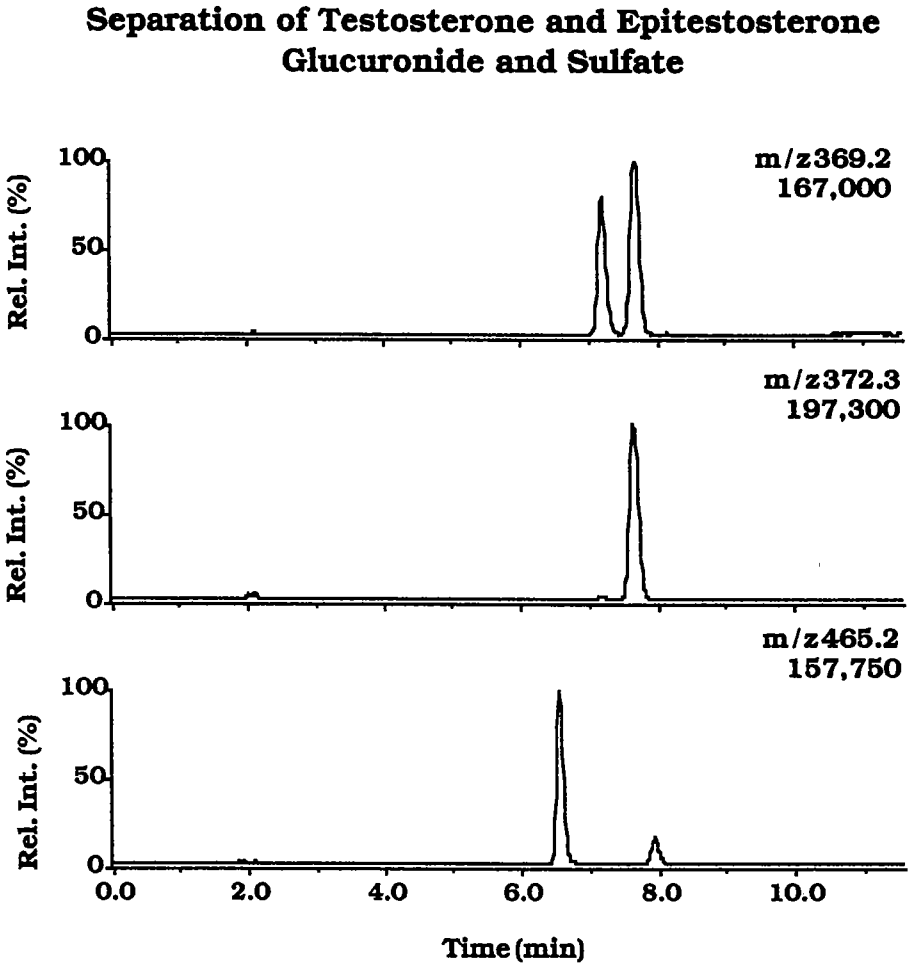

Figure 2. HPLC/MS SIM separation of testosterone and epitestosterone sulfate (m/z 369 panel) d_3-epitestosterone sulfate (m/z 372 panel) and testosterone and epitestosterone glucuronide (m/z 465 panel).