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Some Useful Applications of *Helix Pomatia* Juice in Anabolic Steroid Testing. Essential Metabolites of Sulfate Fraction.

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Introduction

Athletic drug testing laboratories are continually improving limits of detection in order to find better ways to test for steroids for longer periods after administration. Along with the application of newer and more sophisticated detection technologies, such as high resolution mass spectrometry (1-3), selection of the appropriate metabolite is essential. Earlier excretion studies were concentrated on the most abundant metabolite(s) in combined pools of urine collected over a period of time after drug administration. The metabolites were further studied, their structures elucidated, some of them synthesized and recommended for routine monitoring in screening procedures (4, 5). The later findings revealed long term excreted metabolites with slower excretion rates (6-11), such as methandienone metabolites: epimethendiol and 18-normethenol (6-10); 4-chloro-1,2-dehydro-17α-methyltestosterone metabolite: 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one (11); mestanolone metabolites: 17β-methyl-5α-androstan-3α,17α-diol and 17,17-dimethyl-18-nor-5α-androst-13-en-3α-ol (9); and a clostebol metabolite: 4ξ-chloro-3β-hydroxy-5α-androstan-17-one (5). Interestingly, long term metabolites involve sulfatation step at some stage of their formation.

Steroids are excreted in urine mainly as β-glucuronides and sulfate esters, which are not suitable for gas chromatographic analysis. Cleavage of these conjugates is an essential step in sample preparation procedures. Enzymatic hydrolysis is usually used for deconjugation (12, 13). Depending on the type of the enzyme, glucuronides only can be cleaved (*E. coli*) or glucuronides plus most of the sulfates (*H. pomatia*). Enzymatic hydrolysis must be followed by solvolysis for full liberation of steroids from their sulfate esters (14). Most laboratories perform urinary steroid screening in free and glururonide fractions using *E. Coli* enzyme (4, 5). Metabolites excreted as sulfates usually remain undetected.

Experimental

Steroids and excretion studies
Mestanolone, mesterolone, methandriol and methyltestosterone were purchased from Sigma (St. Louis, MO). Excretion studies were performed with healthy male subjects after single oral dose, typically 20 mg. Urine specimens were collected for two week after administration and were stored refrigerated.

Reagents and materials
β-Glucuronidase/arylsulfatase type H-2 (Cat.# G7017) and type H-3 (Cat.# G8885) from *Helix pomatia* were purchased from Sigma. β-Glucuronidase type K12 from *Escherichia coli* was supplied by Fluka (Milwaukee, WI). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Campbell Science Corp. (Rockton, IL), ammonium iodide 99+% from

Aldrich (Milwaukee, WI). C_{18} solid phase (200 mg) extraction cartridges were purchased from Varian (Harbor City, CA).

Urine sample preparation

Procedures and sequence of urine clean up, hydrolysis with *Helix pomatia* or *Escherichia coli* enzymes, solvolysis, and separation of fractions are presented in a flow chart in fig.1. Urine solid phase clean up and extraction each time was performed after priming C_{18} cartridges with 2 mL of methanol and 2 mL of water. *H. pomatia* enzyme was used in acetate buffer (pH 5.2) solution (1 : 0.05 w/w): 1 mL per 4 mL urine extract. *E. coli* enzyme in phosphate buffer (pH 7) solution (1 : 0.05 w/w): 1 mL per 4 mL urine extract. Intermediate 30% acetonitrile wash during solid phase extraction was used to remove contaminants and remaining conjugated steroids.

The dry residues were derivatized with 75 μL of MSTFA/NH_4I/Dithioerythritol (1000:2:3 v/w/w) for 15 minutes at 70°C. Samples were trasfered into vials, 1 μL was injected into the GC/MS.

GC/MS was Hewlett-Packard 5890/5970 with HP 7673A autoinjector and UNIX-Target software. Column: HP-1 fused silica, crosslinked methylsilicon, 16.5m, 0.2 mm i.d., 0.11 μm film thickness. Helium carrier gas was used at linear velocity 40 cm/sec. Injection split ratio was 1:10. Oven temperature program: hold at 180°C for 0.3 min; raise at 3°C/min to 231°C; then 30°/min to 310°C, hold for 1.07 min. Injector temperature was 270°C, transfer line 280°C.

Results and Discussion

Fig. 2 shows testosterone/ epitestosterone fragment of the routine steroid profile performed on a mixture of unconjugated steroid standards after "hydrolysis" with three enzymes. *H. pomatia* type H-3 evidently causes partial degradation of testosterone and epitestosterone, indicated by their reduced peak abundances, and appearance of androstan-3,17-dione. This undesirable side effect was a rationale for replacing this commonly used enzyme with *E. coli*, especially after introduction of the T/E ratio test in 1984. *E. coli* shows no evidence of testosterone and epitestosterone loss and conversion into androstandiones (middle chromatogram, fig. 2). Chromatogram 3 represents a cleaner preparation of *H. pomatia* (type H2) enzyme without side effects. This preparation was used throughout the study. New batches were checked for the absence of degradation activity with standards. Urine matrix noticeably inhibits enzymatic activity of *H. pomatia* (12, 13). Therefore, initial solid phase removal of urine is important. *E. coli* did not show difference between hydrolysis in urine and in extracted material (15).

Successful separation of urinary endogenous steroid conjugates is demonstrated in fig. 3. The upper chromatogram represents glucuronide fraction cleaved by *E. coli.* The second chromatogram shows sulfate fraction steroids liberated by *H. pomatia* from remaining conjugates. The third chromatogram is a result of *H. pomatia* hydrolysis of another portion of urine extract and it contains all glucuronides and most of the sulfates. At the bottom are residual sulfate fraction steroids liberated by solvolysis. 3β-Hydroxy steroids, such as DHEA, epiandrosterone and 5-androsten-3β,17β-diol, appear only in sulfate fraction, fully cleaveable by *H. pomatia*. As the bottom chromatogram shows, sulfates of 3α-hydroxy-5α- steroids, such as

androsterone, can only be cleaved by solvolysis (12, 14). Fig. 3 shows "typical' steroid profiles. Individual differences in relative excretion of sulfates and glucuronides are known (16) and they may be significant.

Application of this procedure to the excretion urines of some synthetic anabolic steroids yields interesting findings. Mestanolone excretion profiles in 16 hour urine are shown in fig. 4. Glucuronide fraction on the upper chromatogram is represented by well known (5, 6) 3α-hydroxy metabolites: 3α-hydroxymestanolone and 3α-hydroxy-17-epimestanolone. Remaining sulfate fraction (the second chromatogram) reveals two interesting peaks with mass spectra similar to above 3α-hydroxymestanolone and its 17-epimer, however, with greater retention times. This is consistent with 3β-hydroxy structures. Based on mass spectra, retention times and sulfate conjugation, these new metabolites are identified as 3β-hydroxymestanolone and 3β-hydroxy-17-epimestanolone. Reduction of the 3-keto function into 3β-hydroxy has not been previously reported for any 17-methyl-17-hydroxy steroid. Solvolysis yields additional 3α-hydroxy metabolites from the sulfate fraction, which constitutes more than 50% of the amount found usually in routine screening in the glucuronide fraction. Later excretion of mestanolone is characterized by the rapid decline of "major" 3α-metabolites. 3β-Metabolites, especially 17-epi, stay longer in the body. The latter is easily detectable on the seventh day of excretion in full scan mode. Four isomeric 3-hydroxy metabolites are shown in fig. 5.

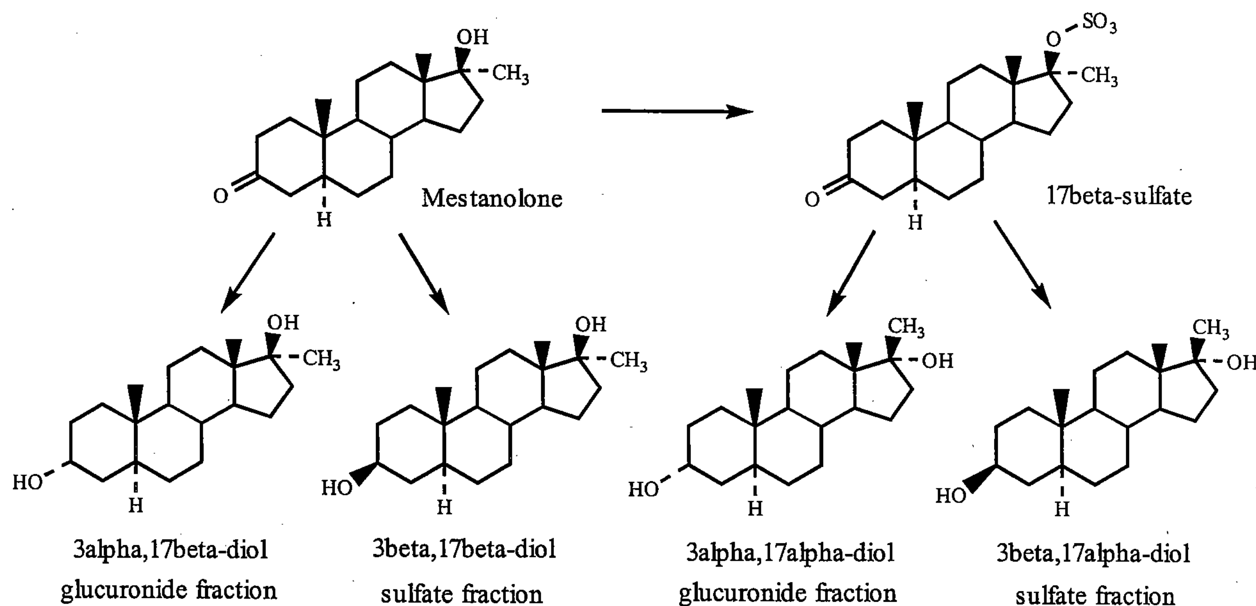

Figure 5. 17-epimerization, 3α- and 3β- metabolic reduction of mestanolone.

These four metabolites were also found in urine after administration of methyltestosterone. Lower concentrations of 3β-hydroxy metabolites are due to the preferential 5β metabolic pathway, which does not allow 3β-hydroxy reduction to occur (5). Nevertheless, 3β-hydroxy-5α- metabolites, hardly noticeable in early excretion, become apparent in the later ones. Methandriol follows the same path (fig. 6) via intermediate methyltestosterone (4, 5). Methandriol's distinctive metabolites can be found only in the form of sulfates: parent (4) and the 17-epimer. The latter, found in this study, is a long term metabolite. The distinction between

methandriol and methyltestosterone steroid profiles, therefore, can only be made by sulfate fraction analysis.

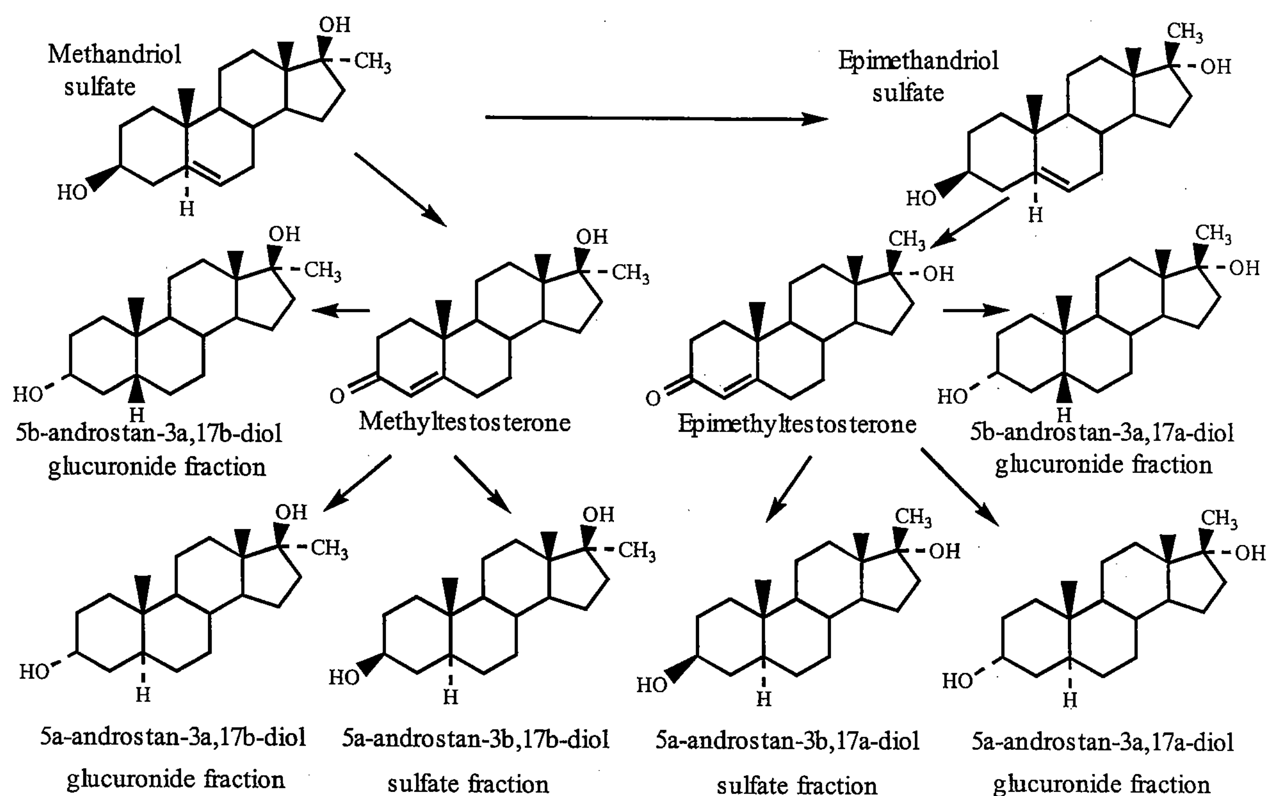

Figure 6. Metabolites of methandriol and methyltestosterone.

Mesterolone metabolites were also studied in the sulfate fraction because the pre-existing 5α-configuration of the parent steroid makes 3β-hydroxy reduction and consequent sulfatation possible. Indeed, 3β-hydroxy-1α-methyl-5α-androstan-17-one was found as a stable long term metabolite in the sulfate fraction, fully cleaveable by *H. pomatia*. It can be detected in urine over 10 days after a single oral ingestion; longer than can the regular 3α-hydroxy metabolite. Routine detection of this 3β-hydroxy metabolite proved very successful in our laboratory. Mass spectrum is similar to 3α-hydroxy isomer, retention time is higher (elutes between epitestosterone and testosterone).

Application of *H. pomatia* for deconjugation of sulfates also extends the detection window for such practically important steroids as methenolone. 16β-hydroxymethenolone sulfate is a metabolite of choice for routine screening, since it stays longer in the body than the others.

Conclusions

Helix pomatia enzyme cleaves both glucuronides and most of sulfate steroid conjugates in urine.
Side effects of *H. pomatia* can be minimized.
Contribution of the sulfate fraction in steroid profiles is significant.

Elimination rates of steroid sulfates from the body are lower than those of glucuronides. Inclusion of sulfates into steroid screen improves detection retrospectivity.

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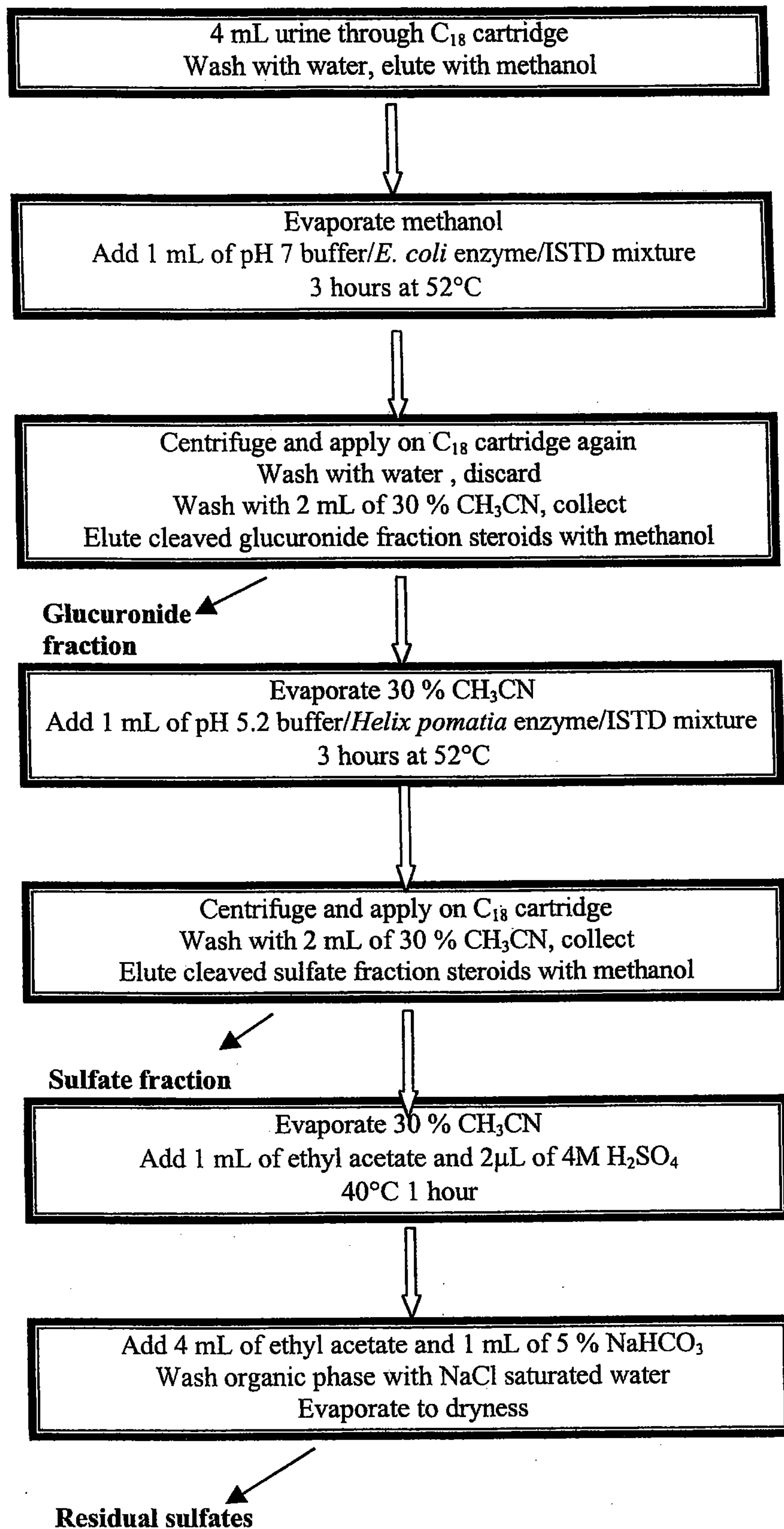

Figure 1. Consecutive solid phase extraction of urine, separation of steroid conjugates, hydrolysis and solvolysis Flow chart.

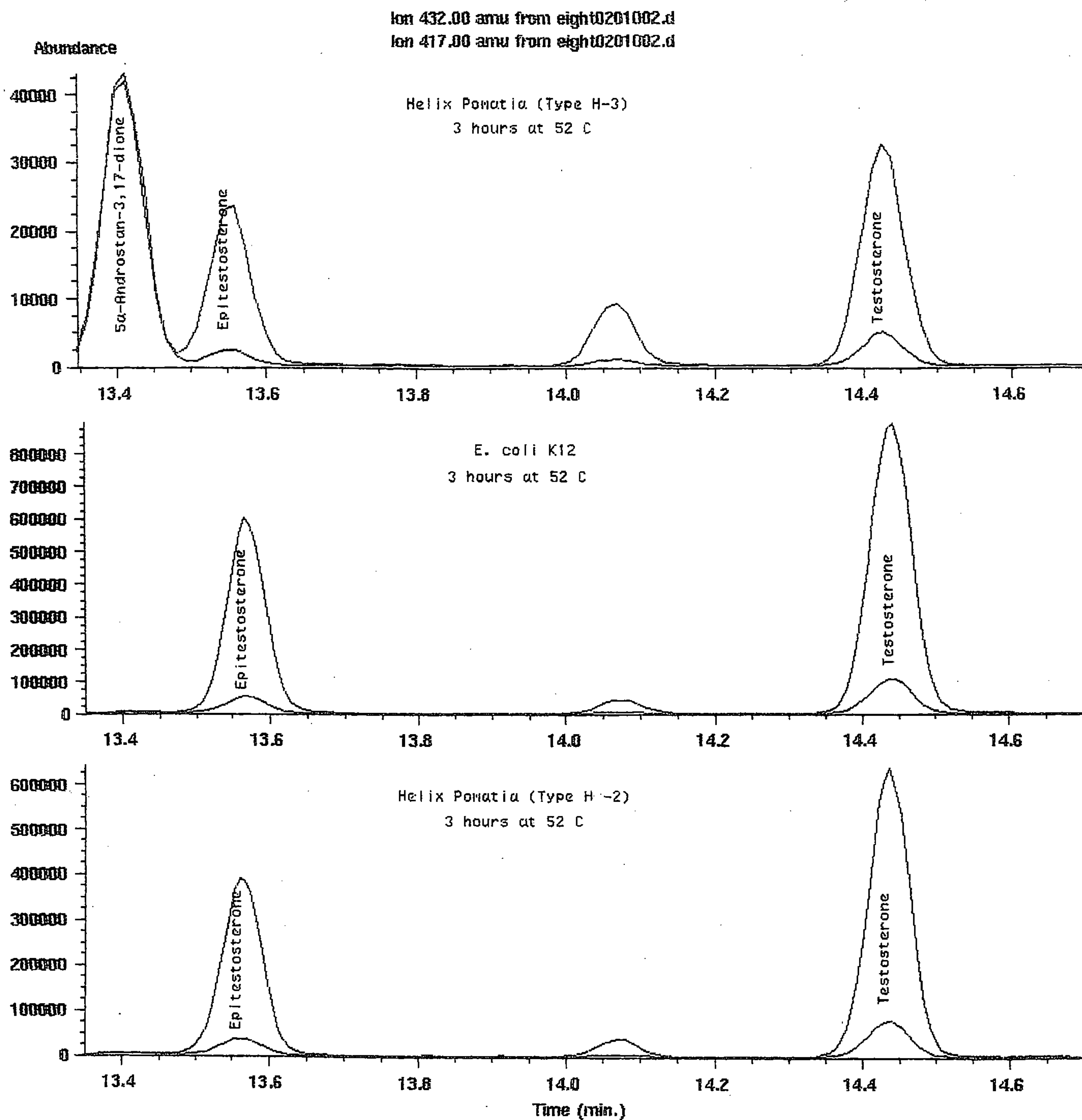

Figure 2. Effect of three enzyme preparations on deterioration of testosterone and epitestosterone and on conversion into androstan-3,17-diones.

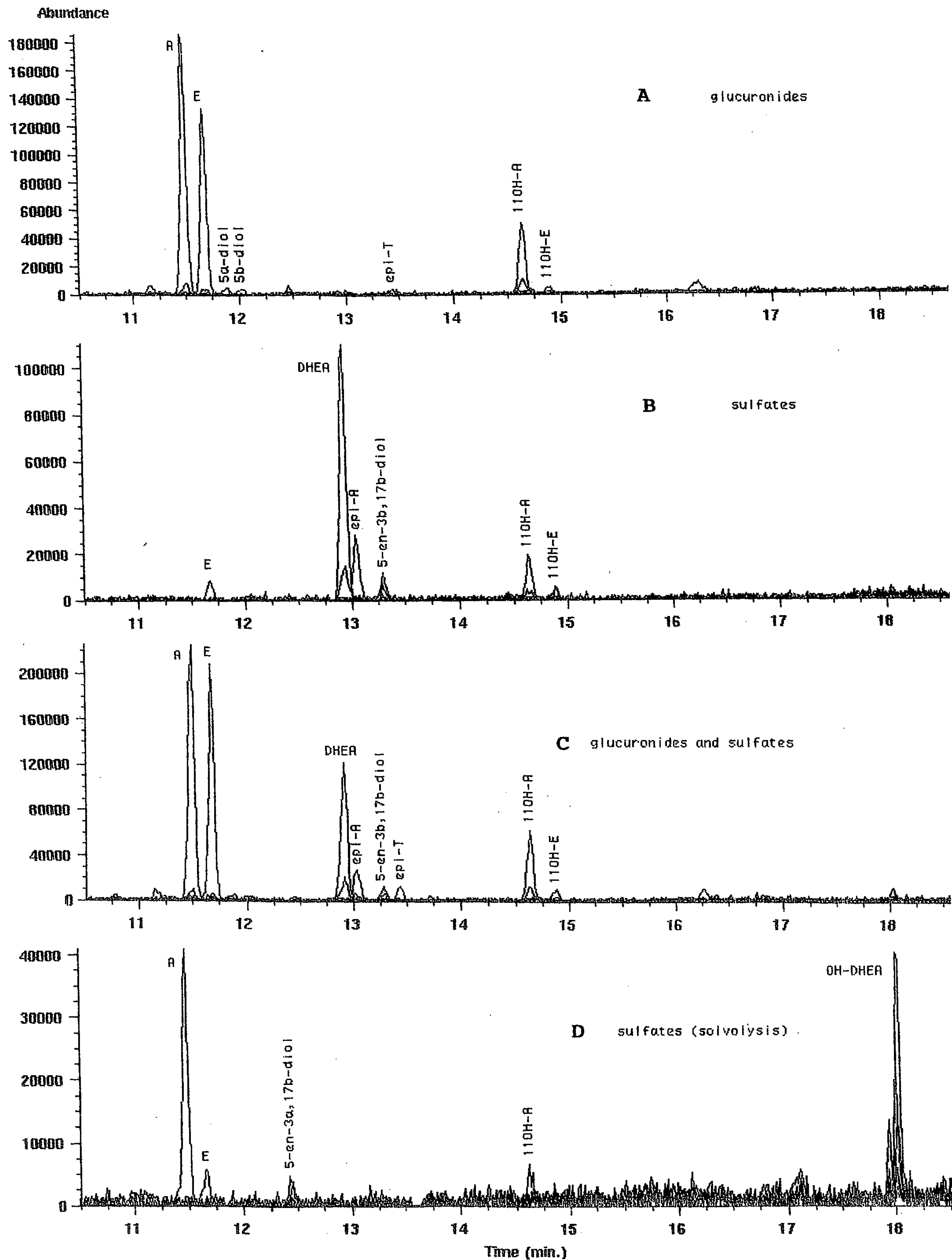

Figure 3. GC profiles of endogenous urinary steroids, excreted as: **A** - glucuronides (hydrolysis with *E.coli*), **B** sulfates (hydrolysis of remaining conjugates with *H.pomatia*), **C** – glucuronides and sulfates (hydrolysis with *H.pomatia*), and **D** – remaining sulfates (solvolysis).

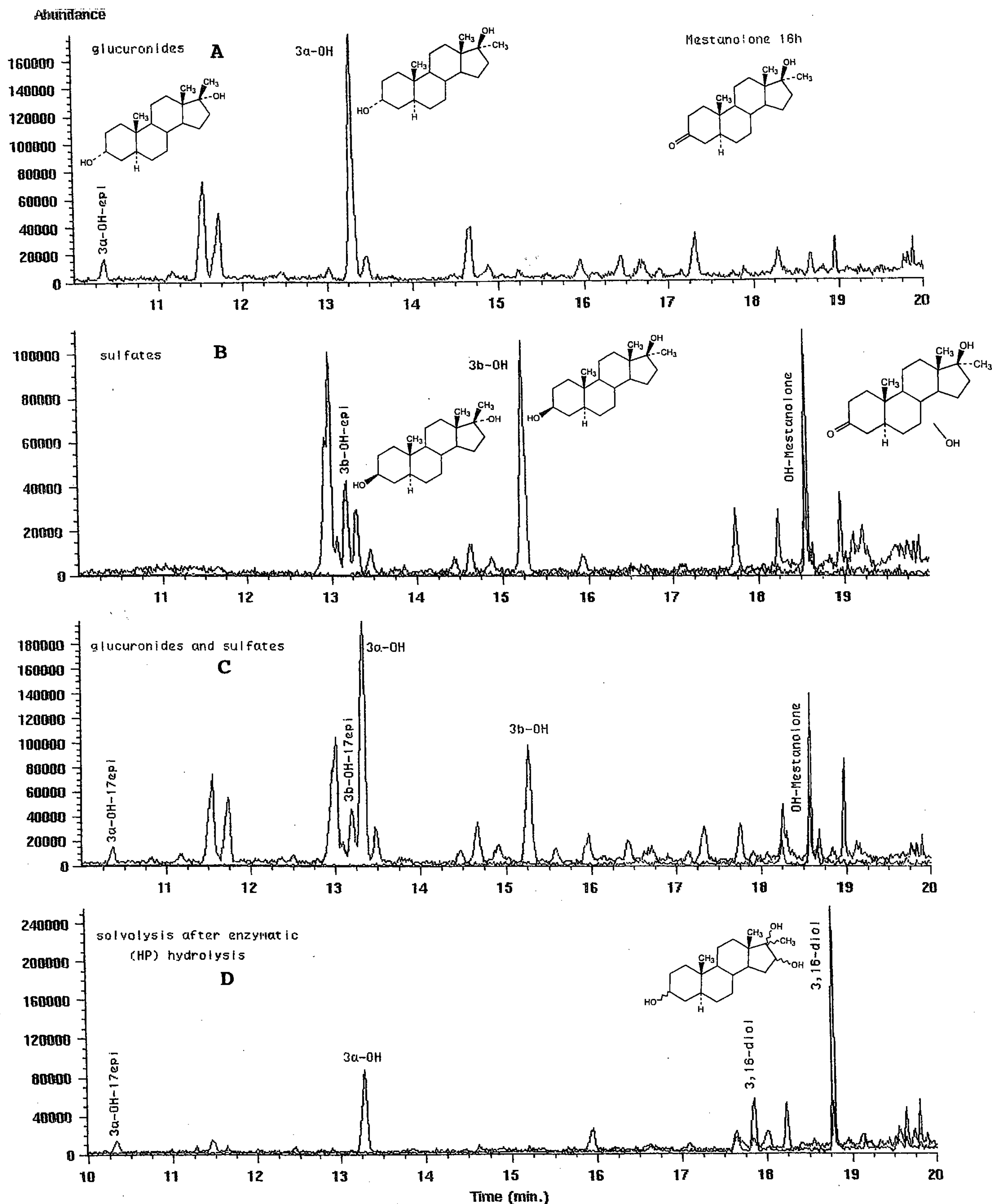

Figure 4. Mestanolone metabolites in urine (16 hours after oral 20 mg administration): **A** – glucuronides, **B** – sulfates, **C** – glucuronides and sulfates, **D** – remaining sulfates.