

Demonstration of 19-Nor-Dehydroepiandrosterone (19NorDHEA - *NorAndrost-4-ene-3 β -ol,17-one*), 1-DHEA (CAS Registry No.: 510-64-5), 1,4,6-androstatriene-3,17-dione (ATD, CAS Registry No.: 633-35-2) and 4-Dehydroepiandrosterone (4-DHEA, CAS Registry No.: 571-44-8) in the urine of a normal Boar

M. Mychajlonka, Ph. D., Lab. Dir., Food Safety, LLC

ABSTRACT

Of the many steroids known to be present in uncastrated boars, this study substantiates the presence of four additional steroids which have heretofore not been described as present. These are: 1,4,6-androstatriene-3,17-dione (ATD, CAS Registry No.: 633-35-2); 4-Dehydroepiandrosterone (4-DHEA, CAS Registry No.: 571-44-8); 1-DHEA (CAS Registry No.: 510-64-5) and 19 Nor DHEA.

INTRODUCTION

The Dietary Supplements Health Education Act of 1994 states that any material that can be shown to have already been in the food supply at the time this law was passed is, by virtue of such demonstration, to be considered a natural and normal constituent of food and cannot therefore be considered either an adulterant or an additive whose presence in food requires safety testing.

METHODS

This study was done in two parts. The first test subject was a single, mature, fully-grown, uncastrated boar resident on the grounds of the Agricultural College of Michigan State University. The repeat study made use of two boars (at the same institution) unrelated to the first one (or to each other) and whose urine was gathered in the same way.

The animals were followed over a period of some five days and its urine was caught in mid-stream in a specially designed container fastened to a long extension pole. All animals were reported to be unconcerned by the process of collecting their urine in this way and so under no observable stress. Recovered urine was kept cold during the time the complete sample (2 liters) was being accumulated and also kept cold prior to and during processing.

The pH of the urine was adjusted to a pH of about 6.0 and then centrifuged at 2,000 x g to remove sediment and debris that would otherwise clog the absorbent columns used. The columns used were manufactured by Honeywell Burdick & Johnson and were polypropylene columns (Product: 9008) having an internal volume of 8 ml each of which contained 1000 mg of C-18 adsorbent trapped between microporous frits. These were the largest capacity C-18 columns commercially available. The two liters of sample were loaded onto three such C-18 columns (in the case of the single animal tested first) and onto 15 of the same type and capacity SPC columns in the case of the repeat. For an unexplained reason, the urine sample from the second two boars seemed to clog the SPC columns to a greater extent than did the urine from the first animal. This necessitated the use of more SPE columns for the repeat experiments.

All three columns (for the first run) were fastened to a solid phase extraction (SPE) manifold that was itself connected to a chemically-resistant, reciprocating vacuum pump. Each column was activated

with methanol, washed with distilled water and then loaded with approximately 600 ml of clarified and pH adjusted boar urine. After loading, each column was air dried before being conducted to the analytic lab.

All samples (both first and second runs) were analyzed in a Hewlett-Packard model 5890 gas chromatograph fitted with an auto-sampler and coupled to a Hewlett-Packard model 5972 mass spectrographic detector. Two microliters of sample were injected, in split-less mode, into an injection module held at 225° C. The column used was capillary column (27 meters in length and 0.32 mm in inner diameter) carrying a 0.5 µm thick layer of DB-5. The analytic run was conducted using a temperature program consisting of a two minute hold at 90° C followed by heating at 10° C/minute up to a final temperature of 310° C. Each run was conducted using helium carrier gas at a flow rate of 1.3 ml/minute. The mass spectrographic detector was set to conduct 2.6 scans per second between the limits of 29 and 470 atomic mass units (AMU).

A variety of elution procedures were tested in the laboratory. The reason for this was the rather unusual nature of the ATD molecule. The premise under question was whether the possession of three double bonds and two keto groups might not make this steroid considerably more hydrophilic (*i.e.*, more soluble in water) than might be the case for some of the more familiar steroids. A preliminary study was conducted which sought to demonstrate only the presence of ATD. A second study was then performed to repeat the ATD results and to extend these to another steroid, 4-DHEA.

For the first study, each cartridge was rinsed with two milliliters of distilled water. The water eluates were themselves extracted with methylene chloride, dried down and the residue dissolved in 0.25 ml of 20% methanol and 10% acetone in toluene to see if any ATD was present in the water rinse. Each SPE column was then sequentially eluted twice each with two milliliters of: methyl tert-butyl ether (MTBE), methylene chloride (MC), and then methanol. The combined 4 ml of each of the solvents were collected in separate 13mm x 100mm tubes. Each combined eluate was evaporated to dryness under a stream of nitrogen gas while being warmed in a water bath to 35° to 40° centigrade. The residue from the MTBE eluates was light brown. The residue from the MC eluates was bluish gray. The residue from the methanol was both brown and copious.

The residue from each solvent was taken up in 0.25 ml of 10% acetone in toluene and analyzed by GC-MS. After these analyses were completed, the remaining elutions were all combined, evaporated to dryness again and processed (as described below). The dried samples were taken up in 0.25 ml of 20% methanol and 10% acetone in toluene. This combined sample was then analyzed to obtain the final result.

As a recovery control, one hundred milliliters of a human urine sample was spiked with a known amount of ATD and applied to a conditioned C-18 column. This column, containing a spiked sample, was then eluted twice with two milliliters of MTBE, MC and methanol. Each solvent was pooled and evaporated to dryness then reconstituted with 0.25 ml of 10% acetone in toluene and analyzed by GC-MS.

Another series of human urine samples (10 ml each) were spiked with ATD and directly extracted by liquid/liquid extraction in 15 ml test tubes with MTBE, MC, acetonitrile (after saturating the urine sample with sodium chloride) and ethyl acetate. The solvent layers were removed after centrifugation, combined and each passed through sodium sulfate drying columns. Each sample was then evaporated to dryness under nitrogen and taken up in 0.25 ml of 10% acetone in toluene. Each sample representing a different liquid/liquid solvent extraction system was then analyzed by GC-MS.

The repeat experiment followed essentially the same extraction protocol. However, since it was previously discovered that at least some ATD was seen in the water washes of the SPE column, this water wash was omitted for the repeat experiment. Instead, each SPE column was extracted, three



Fig. 1



Fig. 2

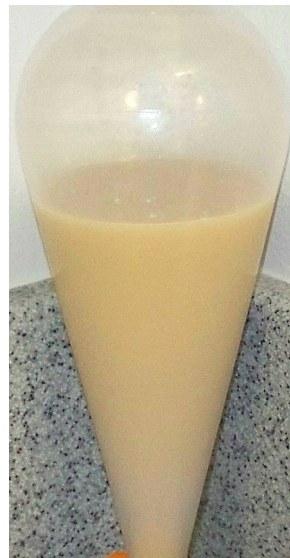


Fig. 3



Fig. 4

times in succession, with 1.0 ml of methanol and all 45 ml of the methanol extract were combined into a Teflon separatory funnel (Fig. 1). This funnel was held over (but not immersed in) a 55°C water bath. The escaping water vapor served to heat the separatory funnel well enough to allow for evaporation of the methanol at a reasonable rate. The contents were frequently tipped so as to distribute the sample over the maximum interior surface area of the funnel and the contents evaporated to dryness (Fig. 2).



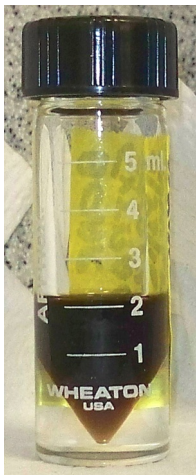
the total

Fig. 5



Fig. 6

Thirty ml of room temperature, 0.5M, pH 6.0 sodium acetate buffer was added to the dried residue in the funnel. The funnel was then secured in a tumbler rotating at 60 rpm and tumbled end over end for approximately six hours. At the end of this time, the sample in the form of a very even suspension (Fig. 3). Two successive liquid/liquid extractions using 30 ml of methylene chloride were performed. The contents of the funnel were mixed intermittently at room temperature for one hour. Fig. 4 shows the first of these. The methylene chloride layer was removed using the valve of the separatory funnel. However, it appeared that a fairly stable emulsion had formed during the methylene chloride extraction (Fig. 5). To extract the maximum amount of sample, this emulsion was broken by centrifugation for one hour at 2,500 x g in a swinging bucket centrifuge (Fig. 6). The extra methylene chloride recovered from the emulsion was added to collected.



The total recovered methylene chloride fraction was concentrated in a cone bottom tube by evaporation under nitrogen. The concentration of sample was stopped when the residual volume reached 2 ml. The methylene chloride extract pictured in Fig. 7 was considered to be representative of the entire two liter sample of urine coming from two different boars.

Fig 7

RESULTS and DISCUSSION

Under the conditions of liquid/liquid extraction, methylene chloride was found to be the best extraction solvent for ATD. Consequently, methylene chloride was used to recover ATD from any aqueous mixture.

Most of the ATD was recovered from the C-18 SPE columns in the methanol eluate. Consequently, there seems little, if any, value in eluting columns with MTBE, MC or ethyl acetate as described in some reports in the literature. However, the methanol eluates, while most efficient at extracting ATD were also quite congested with other co-extracted material. Given the results of the liquid/liquid extraction experiments, it seemed reasonable to elute the SPE columns with methanol and then dissolve the methanol eluates in aqueous sodium acetate buffer (0.5 M, pH 6) prior to performing a liquid/liquid extraction with methylene chloride. This step eliminated other compounds that might interfere with analysis via GC-MS although the overall extraction efficiency observed with spiked samples was somewhat disappointing. The total recovery of ATD using this technique on spiked urine was 54% in one trial and 72% in a replicate trial.

Once the extraction protocols were worked out, the extraction of the SPE columns containing boar urine could proceed. A most surprising finding of this study was that ATD was found in all fractions eluted from the SPE columns, even in the water rinses! The methanol fraction was dissolved in sodium acetate buffer (0.5 M, pH 6) prior to undergoing two sequential liquid/liquid extractions with methylene chloride. The MC extractions were then combined with the other solvents tried then evaporated to dryness. The residue was then redissolved in a solvent mixture consisting of 20% methanol, 10% acetone and toluene prior to being analysed by GC-MS. ATD was quantified using external standards normalized against an internal standard. The ATD also found in the water rinses was quantified separately.

The most abundant steroids in the methanol elution fraction of the boar urine sample under study were: prasterone, androsterone, epiandrosterone, etiocholanolone as well as many others.

This analysis successfully determined the presence of ATD in the sample without glucuronidase hydrolysis and/or solvolysis. It is apparent that boar urine contains many free steroids. Nevertheless, ATD was sufficiently concentrated relative to nearby co-eluting steroids that a definitive mass spectrum was obtained for ATD at the expected retention time, confirming the unquestionable presence of ATD

in boar urine.

The peculiar “hydrophilic-like” solubility properties of ATD found during the course of this study may also explain why the literature is full of reports wherein routine screens for steroids very often found 1,4-androstadiene-3,17-dione (ADD) but missed ATD. The ATD analyte may simply have been removed (and discarded) by extraction protocols focused upon the “real” steroids, known to be immiscible in water.

Lastly, it is worth noting that the average extraction efficiency of ATD was seen to vary between 54% and 72%. A better ATD-specific extraction procedure might well show the presence of ATD even more clearly than this study, which is already definitive.

The boar urine sample showed the presence of both 19-Nor DHEA and 1-DHEA as strong, unambiguous peaks both approaching the one part per million (ppm) level (in boar urine that had been concentrated 1,000X). A problem developed in that with the temperature ramp being used, we could not reliably differentiate between 1-DHEA and 4-DHEA. Therefore, measurement of 4-DHEA was done with a different program, where the temperature gradient was ramped up rapidly to slightly below the temperature needed to release 4-DHEA and then ramped up much more slowly so as to quantify the 4-DHEA moiety. This strategy sufficed to allow the measurement of 1-DHEA and 4-DHEA separately.

This method was repeated twice and both times showed the presence of 4-DHEA in boar urine. Two different analyses showed that standard 4-DHEA eluted with a retention time of 18.948 and 19.428 minutes, respectively in this system. Likewise duplicate runs of the boar urine sample showed retention times of 18.886 and 19.382 minutes. In addition, an analysis of the mass spectral data showed that both the standards and the samples five m/z peaks in common, strongly suggesting identity between the 4-DHEA standard and the peaks with a similar retention time found in the boar urine.

The significance of this study is that four unusual steroids were demonstrated to exist in urine of normal, untreated boars.