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A Screening Method for Synthetic Testosterone and its Precursors based on GC/C/IRMS

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Abstract

We investigate a possible screening procedure for synthetic endogenous steroids based on gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The basic concept is to obtain $^{13}C/^{12}C$-ratios for all relevant steroids in one measurement and to avoid time consuming cleaning steps as far as possible. This could be achieved by selective derivatization of ketonic steroids (androsterone, etiocholanolone) with O-ethylhydroxylamine (OEHA). The resulting ethoxims show a sufficient shift in retention times so that the otherwise coeluting androstandiols can be separated chromatographically. The method is limited by the observed high levels of background, allowing only rough interpretation of the obtained $\delta^{13}C$-values.

1 Introduction

The measurement of $^{13}C/^{12}C$-ratios allows the discrimination of synthetic and natural endogenous steroids [1, 2, 13]. The method usually employed for this purpose is gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). It requires excellent chromatographic conditions, such as baseline separation of the relevant peaks and extreme high purity of the material analyzed [10].

While chromatographic performance is limited mostly by hardware restrictions [7], the latter of the requirements mentioned can be met by HPLC cleanup [9] in the field of steroid analysis. The use of liquid chromatographic techniques makes it very difficult to employ GC/C/IRMS as a routinely used screening procedure. This step is very time consuming and has to be performed with great care because it may effect significant isotopic fractionation [6].

Because the algorithm used to calculate the δ^{13}C-values assumes constant background over time [8, 11] measurements of isotope ratios by GC/C/IRMS are performed best at isothermal conditions [6]. This usually decreases peak quality and chromatographic resolution and thus further restricts the method.

In this study we propose a rapid screening procedure that allows to gain hints for the application of synthetic endogenous androgens and

- does not make use of HPLC,
- does not require isothermal GC conditions,
- and that allows to obtain δ^{13}C-values for etiocholanolone (E), androsterone (A), 5β-androstane-3α, 17β-diol (5βA3αD), 5α-androstane-3α, 17β-diol (5αA3αD), and for at least one of the "internal references" pregnandiol (PD) or pregnantriol (PT).

2 Methods

Chemicals All chemicals were of analytical grade. Potassium hydroxide, sodium acetate and acetic acid were obtained from Merck (Merck, Darmstadt, Germany), OHEA and isopropanol were obtained from Aldrich (Sigma-Aldrich, Weinheim, Germany).

Isolation of Steroids The preparation of urinary samples generally followed the protocol for "Screening IV" described elsewhere [5]. 5ml of urine were prepared. No internal standards were added. The obtained urinary residual was dissolved in 1ml of KOH (10N). The steroid fraction was extracted with 5ml of n-pentane and evaporated to dryness.

Derivatisation Ketonic steroids were derivatized with O-ethylhdydroxylamine (OEHA, 10% in sodium acetate buffer, pH 4.4, 1h, 60 °C).

GC/C/IRMS-Detection The derivatized steroids were dissolved in 30μl isopropanol. 1.5μl were injected into the GC/C/IRMS device. The GC was a model 5890 (Hewlett Packard, Palo Alto, USA). The isotope ratio mass spectrometer was a delta C (Finnigan

MAT, Bremen, Germany). Devices were coupled by a combustion interface II (Finnigan MAT). The reduction oven included in the latter device by default was unmounted.

GC parameters:

- Column: J&W DB35-ms, 15m, 0.25mm ID, 0.25μm film
- Temperature: 60 °C for 1.5min, 40 °C/min to 210 °C, 8 °C/min to 280 °C, 40 °C/min to 310 °C
- Mode: Splitless
- Carrier gas: Helium
- Headpressure: 40psi

δ^{13}C-values were calculated *vs.* PDB[1] *via* CO_2-standard gas (δ^{13}C= -3.66 $^0/_{00}$) according to Craig [4] by vendor provided software (ISODAT 5.3). Two pulses of standard gas (500s–520s, 840s–860s) were used to perform the calculations. Background mode was set to "dynamic".

Corrections for oxygen isotopes were made according to Santrock *et al.* [12].

δ^{13}C-values of derivatized substances were corrected using a mass balance equation [3] where $\delta^{13}C_{OEHA} \approx -31.6\ ^0/_{00}$.

Urine Samples A reference urine collected from eight drug free caucasian males, ten routine samples and a urine obtained from a excretion study with 5α-dihydrotestosterone (DHT) were analyzed.

During the excretion study 25mg of DHT were administered sublingually to a caucasian male (182cm, 76kg). The sample was collected 56h after administration.

The parameter investigated was the difference of δ^{13}C-values between PD and one of the androgen metabolites E, A, 5αA3αD or 5βA3αD ($\Delta_{\delta^{13}C}$). Whenever possible, *i.e.* when of sufficient abundance, the androstanediols were prefered because the only source of urinary 5αA3αD and 5βA3αD is the metabolism of androgenes.

[1] Pee Dee Belemnite, $^{13}C/^{12}C = 0.0112372$

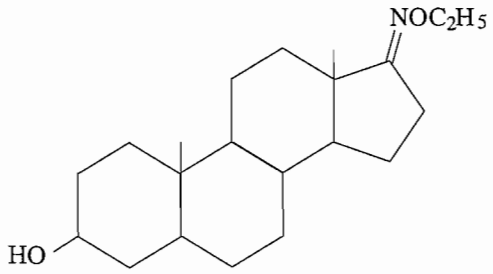

Figure 1: Reaction product of E or A with OEHA.

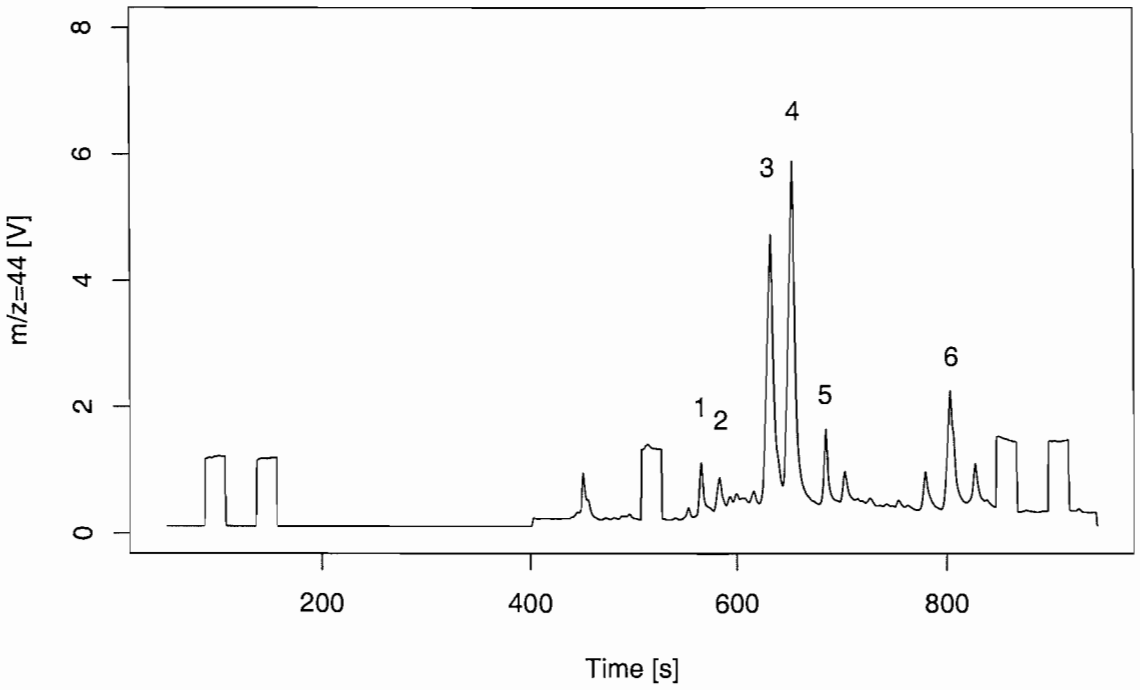

Figure 2: Typical chromatogram obtained from unsuspicous sample. Complete run. Legend: 1, 5βA3αD; 2, 5αA3αD; 3, E-Ethoxim; 4, A-Ethoxim; 5, PD; 6, PT.

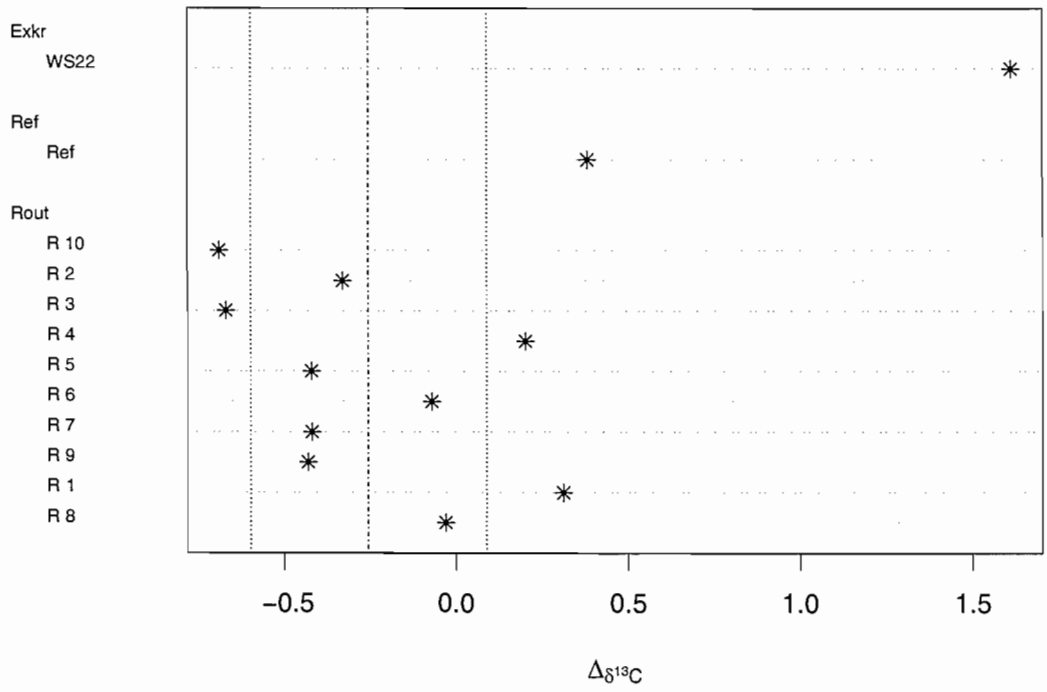

Figure 3: Differences of δ^{13}C-values of androgen metabolites and PD. Legend: Exkr, DHT excretion study; Ref, reference urine; Rout, unsuspicous routine samples; WS22, R1–R10, identities of samples; Vertical lines, $\bar{x} \pm s$ calculated without sample WS22.

3 Results and Discussion

Figure 1 shows the derivatisation product of E or A. The substance is also known as known as SCHIFF's base. Only one of the two possible products (*cis-*, *trans*-isomers in pos. 17) could be observed.

Figure 2 shows a typical chromatogram obtained from a routine sample not suspicous of steroid application.

Figure 3 shows the $\Delta_{\delta^{13}C}$-values for all measured samples. The vertical lines indicate the mean and standard deviation obtained from the negative samples.

The ten routine samples were unsuspicous of synthetic androgen application as was concluded from their steroid profiles (all relevant steroid ratios and concentrations fell well within reference limits).

The value of sample WS22 was calculated from 5αA3αD and PD. It deviates from the mean of the negative samples by more than two standard deviations.

Although it is clearly possible to identify sample WS22 as suspicous, $\Delta_{\delta^{13}C}$ is far lower than expected as the δ^{13}C-values of synthetic DHT and endogenous steroids are approximately -30 ‰ and -24 ‰ respectively. This example clearly emphazises the influence of background in GC/C/IRMS, as conditions are very weak with respect to 5αA3αD (see figure 2). On the other hand WS22 was collected 56 hours after administration, which is a considerable time span.

It does not seem useful to treat urine samples prepared *via* "Screening IV" without further cleanup when one intends to obtain more or less accurate isotope values for a couple of substances. Nonetheless rough information can be gained.

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