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Examination of the kinetic isotopic effect to the acetylation derivatization for the gas chromatographic-combustion-isotope ratio mass spectrometric doping control analysis of endogenous steroids

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In gas chromatographic-combustion-isotope ratio mass spectrometry (GC-C-IRMS) doping control analysis, endogenous androgenic anabolic steroids and their metabolites are commonly acetylated using acetic anhydride reagent, thus incorporating exogenous carbon that contributes to the measured isotope ratio. Comparison of the endogenous $\delta^{13}C$ of free, mono-, and di-acetylated steroids requires application of corrections, typically through straightforward use of the mass balance equation. Variability in kinetic isotope effects (KIE) due to steroid structures could cause fractionation of endogenous steroid carbon, resulting in inaccurate results. To test for possible KIE influence on $\delta^{13}C$, acetic anhydride of graded isotope ratio within the natural abundance range was used under normal derivatization conditions to test for linearity. In all cases, plots of measured steroid acetate $\delta^{13}C$ versus acetic anhydride $\delta^{13}C$ were linear and slopes were not significantly different. Regression analysis of the $\Delta\delta^{13}C$ of enriched acetic anhydrides versus $\Delta\delta^{13}C$ of derivatized steroids shows that KIE are similar in all cases. We conclude that $\delta^{13}C$ calculated from the mass balance equation is independent of the $\delta^{13}C$ of the acetic anhydride reagent, and that net KIE under normal derivatization conditions do not bias the final reported steroid $\delta^{13}C$. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: isotopic ratio combustion mass spectrometry (IRMS); kinetic isotope effects; acetylation derivatization; doping control analysis

Introduction

Harmonization of gas chromatographic-combustion-isotope ratio mass spectrometric (GC-C-IRMS) analysis of endogenous anabolic steroids of the World Anti-Doping Agency (WADA) accredited laboratories is an important objective.[1] Acetylation derivatization is a common practice in GC-C-IRMS analysis, because of the superior gas chromatographic behaviour of acetylated compounds, but it may give greater variation in the results among the WADA-accredited laboratories since not all laboratories derivatize, and those that do use different reagents and protocols. Acetylation induces a major change [2–5] of the isotopic composition of the analytes due to the introduction of carbons of exogenous origin. This phenomenon is taken into account by mass balance (Eqn. (1)), which corrects for the isotopic interference of acetate group:

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad (1)$$

with n = number of moles of carbon, c = compound of interest, d = derivative group, and cd = derivatized compound.

Several reports[2–5] have shown that acetylation can produce significant isotopic fractionation that has been ascribed to kinetic isotope effects (KIEs), which are associated with the formation of carbon-oxygen bonds during the rate determining step. The mass balance equation (Eqn. (1)) is a general relationship describing isotope ratios of mixed components and does not explicitly account for KIE when applied to reaction mixtures. Isotopic fractionation may be reflected implicitly in calculated $\delta^{13}C$ depending on the mechanism of the reaction. For practical purposes, a correction factor for the derivative group can be calculated according to a rearranged version of Eqn. (1):

$$\delta^{13}C_d = \left(n_{cd}\delta^{13}C_{cd} - n_c\delta^{13}C_c\right)/n_d \quad (2)$$

Considering the possibility of a KIE, $\delta^{13}C_d$ reflects the apparent $\delta^{13}C$ of the derivative group modified for isotopic fractionation, if any. Calculation of the correction factors is performed after analysis of the acetylated and the non-acetylated steroids in conjunction

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with Eqn. (2). For doping analysis,[6,7] various steroids may produce different correction factors if different KIEs apply to each steroid. It is of value then to experimentally evaluate whether the KIE are similar from steroid to steroid. Saudan *et al.*[8] estimated the correction factor for the acetylation of 1-octanol and compared the $\delta^{13}C$ values of underivatized steroids with the corrected values of acetylated steroids with that correction factor and showed that KIEs associated with acetylation were likely to be similar. They noted that while underivatized monohydroxylated steroids and acetylated steroids after correction gave similar $\delta^{13}C$ values, corrected bisacetylated dihydroxy steroids gave less depleted values with respect to underivatized counterparts. Piper *et al.*[9] have presented detailed estimation of correction factors for steroids of doping control interest, finding only small differences with the exception of 11-hydroxyandrosterone.

Possible variability of correction factors affecting the $\delta^{13}C$ of various steroids could, in principle, alter the $\delta^{13}C$ and hence are significant for the comparison of the IRMS results of different steroids. In order to elucidate whether the correction factors are the same or different for different steroids, we studied the influence on the isotopic composition of acetylated steroids that have been acetylated simultaneously with graded, prefixed low-level ^{13}C-enriched acetic anhydride.

Experimental

Materials

5α-androstane-3β-ol (CU/USADA 30-1) with a $\delta^{13}C$ value of −29.7 ‰[10] was used. 16(5α)-Androsten-3α-ol, 5β-androstan-3α-ol-17-one (etiocholanolone), 5α-androstan-3α-ol-17-one (androsterone), 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, 11-ketoetiocholanolone, 4-androsten-17α-ol-3-one (epitestosterone), testosterone, 5β-pregnan-3α,20α-diol (pregnanediol) and 11-hydroxyandrosterone were purchased from Steraloids (Newport, RI, USA). All solvents used were of analytical grade (Labscan, Tech-line, Athens, Greece). The calibration standard (n-octadecane) 0.15 mg/ml in cyclohexane for GC-C-IRMS was purchased from Chiron AS, Norway. Acetic anhydride 99.5%+ and pyridine 99% + were from Sigma-Aldrich, Munich, Germany. Acetic anhydride −1,1′-$^{13}C_2$, 99 atom % ^{13}C was purchased from Sigma-Aldrich, Munich, Germany. Carbon dioxide with 99.7% purity was purchased from Air Liquide, Hellas, Greece and was used as the reference gas. A stock solution in methanol was prepared from the above steroids at concentration of 200 μg/ml. The combustion furnace was purchased from ISOPRIME Ltd, Cheadle Hulme, UK (T4003321 Rev. 2) and was used as it was.

Instrumental conditions

Carbon isotope measurements were performed on an Isoprime IRMS instrument (GV Instruments, Manchester, UK) coupled to an 6890 N gas chromatograph (Agilent, Santa Clara, CA, USA), via a combustion system (combustion interface and combustion furnace, GV Instruments, Manchester, UK, Isoprime Ltd, Cheadle Hulme, UK) and a HP-7683 Agilent auto-sampler. Injections were performed in splitless mode to avoid possible mass discrimination due to differences in volatility. The injector temperature was set to 250 °C and the volume of injection was 2 μl. The splitless valve time period was 1 min. The fused silica capillary column Supelco SPB™-50 (Sigma-Aldrich, Germany) was of 30 m length, 250 μm internal diameter, and 0.25 μm film thickness. Helium of 99.99% purity was used as carried gas and was set to a constant flow of 1.20 ml/min at a column temperature of 70 °C. The initial oven temperature was set at 120 °C and held for 3 min, then increased at 40 °C/min to 280 °C and held for 10 min, then increased at 40 °C/min to 300 °C and held for 2 min. The run time of the chromatographic analysis was 27.50 min.

The outlet of the GC column was connected to a combustion furnace. The interface and the furnace temperatures were set to 350 °C and 850 °C, respectively. The combustion gases, CO_2 and H_2O, passed through a capillary made of Nafion, for water removal. Two reference carbon dioxide gas pulses were introduced in each analysis.

The mass spectrometer electron impact ion source was used with an ionization current of 400 μA and the masses *m/z* 44, 45, and 46 were monitored by three independent Faraday ion collectors. A Masslynx data system, version 4.0 was used for analysis and data evaluation. Finally, all the $\delta^{13}C$ values were reported relative to the Pee Dee Belemnite (PDB) international isotope standard.

Acetylation of steroids

For the acetylation of steroids 50 μl of the stock solution were evaporated to dryness under a stream of nitrogen at 50 °C and then incubated at 60 °C for 1 h with 100 μl acetic anhydride and 100 μl pyridine. The excess reagents were evaporated under a stream of nitrogen at 50 °C and the residue was reconstituted with 50 μl acetonitrile HPLC grade. Two μl from these solutions were injected into the GC-C-IRMS. For the measurements of the effect of ^{13}C-enriched acetic anhydride, 100 μl of acetic anhydride −1,1′-$^{13}C_2$ was diluted to 10 ml with analytical-grade acetic anhydride. From this isotopic-enriched acetic anhydride solution, diluted solutions of 0.25, 0.50, 1.0, 1.5, and 2.0‰ enrichment in acetic anhydride −1,1′-$^{13}C_2$ were prepared by mixing enriched and analytical-grade acetic anhydride and mixed thoroughly.

Correction factors measurements

For the measurement of the correction factors, 50 μl of each steroid solution at 200 μg/ml was incubated at 60 °C with 100 μl acetic anhydride and 100 μl pyridine. Excess reagents were evaporated under a stream of nitrogen at 50 °C. Moreover, 50 μl of each steroid solution used initially was added again to the corresponding test tube and the solvent was evaporated. No further derivatization occurred, and the residue was reconstituted with 50 μl acetonitrile. Two μl from these solutions were injected into the GC-C-IRMS. This procedure allows the simultaneous determination of the $\delta^{13}C$ values for both the derivatized and the underivatized steroid. For the measurements of primary KIEs, acetic anhydride solutions with 0.25, 0.5, 1.0, 1.5, and 2.0 ‰ enrichment of acetic −1,1′-^{13}C anhydride were prepared by successive dilution of Acetic anhydride −1,1′-$^{13}C_2$ with analytical grade acetic anhydride and mixed thoroughly, as mentioned above (Acetylation of steroids).

Statistical evaluation

Linear plots were evaluated for equivalence of slopes using the Regression Analysis tool in Microsoft Excel 2003 (11.5612.5606) for Windows XP. Slopes for which the 95% confidence limits did not overlap were declared significantly different, corresponding to $p < 0.05$.

Results and discussion

Correction factors were calculated for the steroids of interest after derivatization of ten separate aliquots and duplicate analysis of each aliquot using Eqn. (2). The results are presented in the Table 1. The differences in the correction factors of Table 1 have previously been attributed[2–5] to differences in KIE.

The chromatographic behaviour of some underivatized steroids was problematic and the respective estimation of correction factors may be biased because of tailing or fronting, or subtle overlap.[11] In order to evaluate if the observed differences of correction factors of Table 1 are the result of different KIEs, we studied the acetylation reaction of steroids with ^{13}C-enriched acetic anhydride. It is well known that non-competition KIEs can be evaluated [12–14] at the rate-determining step of the reaction at natural abundance when one of the reactants is in high excess; this is the case for acetic anhydride (AC_2O) in the acetylation reaction. Mechanistically, it has been proposed that in the reaction of acetic anhydride-pyridine system with alcohols (Figure 1) the rate-determining step is the formation of the first acetyl pyridinium ion intermediate through the first tetrahedral transition state (Figure 2).[15] For this reaction, the incorporation of the steroid moiety occurs after the rate-determining step and thus the KIE should be small or constant among different steroids. In contrast, different KIEs for various steroids are expected if the incorporation of the alcohol through the second tetrahedral transition state is the rate-determining step of the reaction (Figure 2). In every case, KIEs are expected to be constant for certain reaction conditions and for particular steroids.

In order to elucidate the effect of acetic anhydride on various steroids, we studied their reaction with graded ^{13}C-enriched acetic anhydride and calculated the $\delta^{13}C$ values of the corresponding acetylated compounds. Linear response was observed for all the tested steroids, which include 16(5α)-androsten-3α-ol, 5α-androstan-3β-ol, etiocholanolone, androsterone, 5β-androstane-3α, 17β-diol, 5α-androstane-3α,17β-diol, 11-ketoetiocholanolone, epitestosterone, testosterone, pregnanediol and 11-hydroxyandrosterone with r^2 better of 0.987 for all the tested steroids (Figure 3).

Table 1. Calculated correction factors (‰)

Steroid	Correction factor (mean ± SD)
5α-androstan-3β-ol	−59.0 ± 1.9
19-noretiocholanolone	−48.0 ± 1.8
Etiocholanolone	−49.8 ± 2.0
Androsterone	−49.5 ± 2.6
5β-androstane-3α,17β-diol	−61.9 ± 2.0
16(5α)-androsten-3α-ol	−55.4 ± 1.5
11-ketoetiocholanolone	−58.1 ± 2.6
pregnanediol	−47.2 ± 1.1

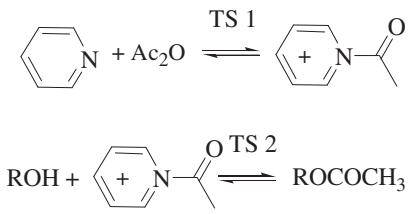

Figure 1. Acetylation reaction of steroids, ROH, with acetic anhydride-pyridine system.

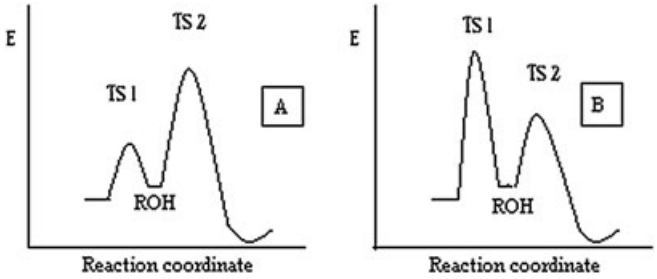

Figure 2. Energy diagrams of the acetylation reaction where the formation of A, the second or B the first tetrahedral intermediate is the rate-determining step.

These results confirm the hypothesis that the KIE associated with the steroid moiety is constant for all steroids, within experimental error. However, the observed linearity cannot differentiate whether the various isotopic compositions of graded acetic anhydrides are the same or different for the various steroids, since the $\delta^{13}C$ values of the steroid acetates depend on the $\delta^{13}C$ values of the underivatized steroids and the $\delta^{13}C$ of the acetic anhydride according to Eqn. 1. To evaluate this matter, we include a KIE term in the part of acetic anhydride correction. We can rewrite Eqn. (1) as:

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_{AC2O} * KIE \quad (3)$$

Furthermore, if we consider the $\delta^{13}C$ values of the derivatized steroids at two different enrichment levels 1 and 2 on ^{13}C of acetic anhydride, Eqn. (3) can be written as:

$$n_{cd}\delta^{13}C_{cd1} = n_c\delta^{13}C_c + n_d\delta^{13}C_{AC2O1} * KIE \quad (4)$$

$$n_{cd}\delta^{13}C_{cd2} = n_c\delta^{13}C_c + n_d\delta^{13}C_{AC2O2} * KIE \quad (5)$$

The difference of Eqns (5) to (4) gives

$$n_{cd}\Delta\delta^{13}C_{cd} = n_d\Delta\delta^{13}C_{AC2O} * KIE \quad (6)$$

Equation (6) shows that the difference of $\delta^{13}C$ values caused from the enrichment of the acetic anhydride, $\Delta\delta^{13}C_{cd}$, is independent of the $\delta^{13}C$ values of the underivatized steroids as well as from the $\delta^{13}C$ of the natural abundance (not enriched) acetic

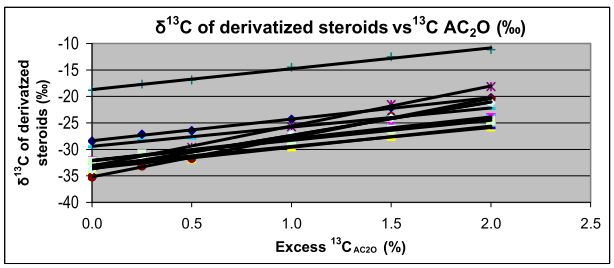

Figure 3. Data and least squares regression plot of $\delta^{13}C$ values of derivatized steroids vs excess of ^{13}C of AC_2O. All responses are linear to $r^2 > 0.98$.

anhydride; it depends only from KIEs that Figure 3 shows are constant for each steroid. Hence analysis of the $\Delta\delta^{13}C$ of the derivatized steroids will show if different KIEs are applied for different steroid acetates. $\Delta\delta^{13}C_{cd}$ values of the corresponding acetylated compounds were plotted against the level of ^{13}C enrichment of acetic anhydride, $\Delta\delta^{13}C_{AC2O}$ and the results are presented in Figure 4 and in Table 2.

The mean slope of all lines is 4.7 ± 1.5 (mean $\pm$ SD) with an RSD of 33%. A statistical analysis of the slopes presented in Table 2, derived from the data of Figure 4, shows that there are two groups of slopes. One is for the monohydroxy AAs and another for the three diol steroids, 5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol, and pregnanediol. A small but non-significant difference is also found for the pregnanediol (C21 steroid) compared to the androstanes (C19 steroid). We hypothesize that these results are in accordance with the different number of acetylated hydroxyl groups, and (non-significantly) the different number of carbons of pregnanediol (C21 steroid) compared to the androstanes (C19 steroids). In order to evaluate this hypothesis, the $(n_{cd}/n_d)\Delta\delta^{13}C_{cd}$ values were calculated and the results were plotted against $\Delta\delta^{13}C_{AC2O}$ according to Eqn. (6).

Figure 5 and Table 3 present these plots. No difference in slopes is found. As can be seen from the results presented in Table 3, the mean slope of different steroid lines becomes tighter, with an RSD of slopes now 6% (39.4 ± 2.2), compared to 33% in Table 2. The slopes are KIE expressed in parts per thousand excess ^{13}C. Using the mean slope, an alternative formulation of $KIE = k(^{12}C)/k(^{13}C) = 1.039$. Notably a plot of the curves that were produced from the mean $(n_{cd}/n_d)\Delta\delta^{13}C_{cd}$ for all the tested steroids, $\pm$ 2SD, (dotted lines in Figure 5) includes curves of all steroids. Residual differences in the observed slopes may be caused by subtle differences in KIE of the different steroids; however, the mean differences are small and non-significant.

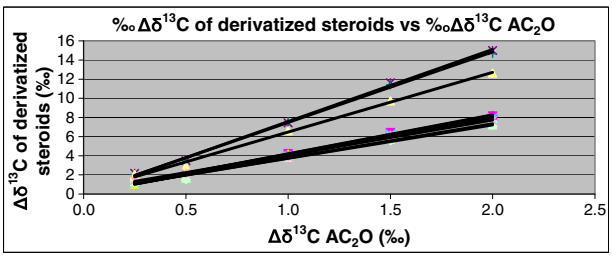

Figure 4. Summary plots of $\Delta\delta^{13}C$ of derivatized steroids against $\Delta\delta^{13}C$ of AC_2O. The slopes of the monohydroxy steroids are significantly different from the slopes of the diols.

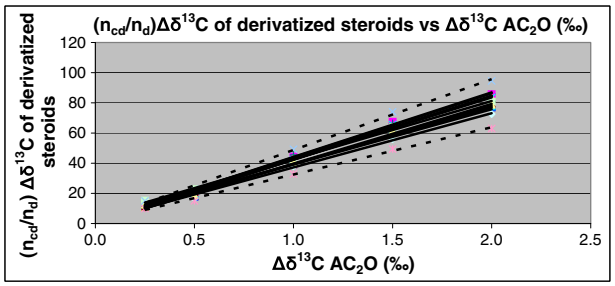

Figure 5. Summary plots of $(n_{cd}/n_d)\Delta\delta^{13}C_{cd}$ of derivatized steroids against $\Delta\delta^{13}C$ of AC_2O. The dotted lines represent the linear regression that emerge from the average of $(n_{cd}/n_d)\Delta\delta^{13}C_{cd}$ responses $\pm$0.2 SD. There are no significant differences in the slopes.

Table 3. Regression analysis of $(n_{cd}/n_d)\Delta\delta^{13}C$ of derivatized steroids vs $\Delta\delta^{13}C$ of AC_2O according to Eqn. (6). There are no significant differences between slopes

Steroid	(ncd/nd)Δδ13C of derivatized steroids vs Δδ13C of AC2O				
	Slope	Intercept	Standard Error	t Stat	P-value
16(5α)-androsten-3α-ol	42.5	1.35	1.2	35.3	0.0000
5α-androstanol	42.8	1.37	1.1	36.3	0.0000
etiocholanolone	42.0	0.30	1.1	38.9	0.0000
androsterone	42.1	0.39	1.3	33.0	0.0001
5β-androstane-3α, 17β-diol	39.6	0.12	1.1	36.3	0.0000
5α-androstane-3α, 17β-diol	38.7	0.53	0.9	41.7	0.0000
11-ketoetiocholanolone	40.6	2.35	2.2	18.5	0.0003
epitestosterone	36.6	2.57	2.4	15.0	0.0006
testosterone	37.5	2.06	1.8	20.7	0.0002
pregnanediol	35.9	1.31	1.3	27.5	0.0001
11-hydroxyandrosterone	38.5	4.23	1.3	29.5	0.0001

Table 2. Regression analysis of $\Delta\delta^{13}C$ of derivatized steroids vs $\Delta\delta^{13}C$ of AC_2O

Steroid	Δδ13C of derivatized steroids vs Δδ13C of AC2O					
	r^2	Slope	Intercept	Standard Error	t Stat	P-value
16(5α)-androsten-3α-ol	0.998	4.05	0.13	0,1	35,3	0,0000
5α-androstan-3β-ol	0.997	4.07	0.13	0,1	33,4	0,0001
etiocholanolone	0.998	4.00	0.03	0,1	38,9	0,0000
androsterone	0.997	4.01	0.04	0,1	33,0	0,0001
5β-androstane-3α,17β-diol	0.998	7.54*	0.02	0,2	36,3	0,0000
5α-androstane-3α,17β-diol	0.998	7.37*	0.10	0,2	41,7	0,0000
11-ketoetiocholanolone	0.991	3.87	0.22	0,2	18,5	0,0003
epitestosterone	0.987	3.49	0.24	0,2	15,0	0,0006
testosterone	0.993	3.57	0.20	0,2	20,7	0,0002
pregnanediol	0.996	6.25*	0.23	0,2	27,5	0,0001
11-hydroxyandrosterone	0.997	3.67	0.40	0,1	29,5	0,0001

* Diol slopes are significantly different from all other slopes ($p < 0.05$) but not different from one another.

Conclusion

This study is strong evidence confirming the approach of Saudan *et al*.[8] for the indirect estimation of one global correction factor for the acetylation reaction with a substance that shows good chromatographic behaviour for both the derivatized and the underivatized substance (1-octanol). The use of low level ^{13}C-enriched acetic anhydride in the acetylation reaction of steroids demonstrates that, at most, only small differences in the KIEs are found and hence that the correction factors for the acetylation reaction are the same for a wide array of steroids of interest to doping control.

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