Journal of Steroid Biochemistry & Molecular Biology 117 (2009) 8–14

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Excretion of endogenous boldione in human urine: Influence of phytosterol consumption

Karolien Verheyden[a,*], Herlinde Noppe[a], Lynn Vanhaecke[a], Klaas Wille[a], Julie Vanden Bussche[a], Karen Bekaert[a], Olivier Thas[b], Colin R. Janssen[c], Hubert F. De Brabander[a]

[a] Ghent University, Faculty of Veterinary Medicine, Research Group of Veterinary Public Health and Zoonoses, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium
[b] Ghent University, Faculty of Bioscience Engineering, Department of Applied Mathematics, Biometrics and Process Control, Coupure Links 653, B-9000 Ghent, Belgium
[c] Ghent University, Faculty of Bioscience Engineering, Department of Applied Ecology and Environmental Biology, Laboratory of Environmental Toxicology and Aquatic Ecology, Jozef Plateaustraat 22, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 30 March 2009
Received in revised form 29 May 2009
Accepted 1 June 2009

Keywords:
Boldione
Phytosterols
Functional-food products
Urine
Human
GC–MS–MS

ABSTRACT

Boldenone (17-hydroxy-androsta-1,4-diene-3-one, Bol) and boldione (androst-1,4-diene-3,17-dione, ADD), are currently listed as exogenous anabolic steroids by the World Anti-Doping Agency. However, it has been reported that these analytes can be produced endogenously. Interestingly, only for Bol a comment is included in the list on its potential endogenous origin. In this study, the endogenous origin of ADD in human urine was investigated, and the potential influence of phytosterol consumption was evaluated.

We carried out a 5-week *in vivo* trial with both men ($n=6$) and women ($n=6$) and measured α-boldenone, β-boldenone, boldione, androstenedione, β-testosterone and α-testosterone in their urine using gas chromatography coupled to multiple mass spectrometry (GC–MS–MS). The results demonstrate that endogenous ADD is sporadically produced at concentrations ranging from 0.751 ng mL^{-1} to 1.73 ng mL^{-1}, whereas endogenous Bol could not be proven. We also tested the effect of the daily consumption of a commercially available phytosterol-enriched yogurt drink on the presence of these analytes in human urine. Results from this study could not indicate a relation of ADD-excretion with the consumption of phytosterols at the recommended dose. The correlations between ADD and other steroids were consistently stronger for volunteers consuming phytosterols (test) than for those refraining from phytosterol consumption (control). Excretion of AED, bT and aT did not appear to be dependent on the consumption of phytosterols.

This preliminary *in vivo* trial indicates the endogenous origin of boldione or ADD in human urine, independent on the presence of any structural related analytes such as phytosterols.

1. Introduction

In 2006, 2% of all human urine samples analysed worldwide by World Anti-Doping Agency (WADA)-accredited laboratories tested positive for prohibited substances. Forty five percent of all these adverse findings were due to the presence of anabolic steroids [1]. This class of drugs contains the male hormone testosterone (T) and several substances structurally related to it, such as boldione (androst-1,4-diene-3,17-dione, ADD) and boldenone (17-hydroxy-androsta-1,4-diene-3-one, Bol) [2] (Fig. 1). Their capability of improving muscle mass and strength has increased the use of anabolic steroids as performance enhancers in human sports [1,3,4]. However, this doping has led to a strict ban on exogenous as well as endogenous anabolic steroids in human sports [2]. Although sophisticated analytical techniques to distinguish between exogenous and endogenous targeted analytes are available [4,5], doping with endogenous steroids remains a serious issue facing doping control agencies [6].

In this context, knowledge on the origin of an analyte is of paramount importance. Boldenone, for example, has long been considered a marker of illegal treatment when detected in urine. However, in extremely rare individual cases, boldenone of endogenous origin can consistently be found at very low nanograms per milliliter levels in urine [2,7]. In contrast, to the best of our knowledge no such evidence exists on the endogenous origin of boldione, the precursor of boldenone [8].

These and other anabolic steroid hormones are characterised by an androstane nucleus consisting of four fused ring structures, three hexane rings and one pentane ring [7,9]. This cyclopentanophenanthrene is also typical of sterols, such as

* Corresponding author. Fax: +32 9 264 7492.
E-mail address: Karolien.Verheyden@UGent.be (K. Verheyden).

0960-0760/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2009.06.001

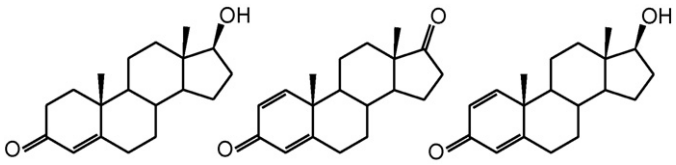

Fig. 1. Chemical structure of different steroid hormones: left, β-testosterone (bT); center, boldione (ADD); right, β-boldenone (bBol).

phytosterols as well as cholesterol, which is the precursor molecule of several anabolic steroids [10–12] (Fig. 2). Phytosterols only differ from cholesterol by their side chain configuration [13–15], allowing them to compete with cholesterol for absorption from the gastro-intestinal tract [15–17]. This characteristic of competition forms the basis for the cholesterol-lowering properties of phytosterols. At a daily concentration of 2–3 g, phytosterols have been shown to reduce plasma cholesterol levels in humans, improving blood lipid profiles and reducing the risk of coronary heart disease [13–15,18,19]. As a result phytosterols, natural constituents of several food products, are also supplemented to food items which are termed functional food [20,21].

In the food industry functional food constitutes the single fastest growing segment [21], but consumers seem to have become more aware of potential food risks such as genetical modification, bacterial contamination, incidence of mad cow disease and the presence of growth hormone residues [22]. Tucker et al. [23] also reported that, already in 2001, the majority of the population in 19 out of 34 countries studied felt that their food is less safe than 10 years earlier. This statement was confirmed in a recent study on the consumers' uncertainty about the safety and quality of their food [24]. Specifically, the potential presence of hormone and veterinary drug residues in food remains one of the major causes of concern to both American [23] and European consumers [24].

Biotransformation of phytosterols to steroid hormones has thus far not been demonstrated in humans while microbial conversion of phytosterols to steroids has been frequently reported [25–31]. In addition, a number of studies has been devoted to the ability of invertebrate organisms to convert phytosterols into anabolic steroids [32–34]. Recently Ros et al. [35] have performed a human study on the excretion of boldenone upon consumption of phytosterol-enriched margarine, however, none of the urine samples tested positive for this anabolic steroid.

The purpose of our investigation was to determine the endogenous origin of several anabolic steroids in human urine, especially boldenone and its precursor boldione. Furthermore, we hypothesized that urinary excretion of these anabolic steroids but also of the main anabolic steroid β-testosterone (17β-hydroxy-androst-4-ene-3-one, bT), its isomer α-testosterone (17α-hydroxy-androst-4-ene-3-one, aT) and its precursor androstenedione (androst-4-ene-3,17-dione, AED) could be associated with phytosterol consumption. In the present study volunteers were asked to consume a phytosterol-enriched yogurt drink at the recommended dose equalling 2 g a day. Subsequently, the influence of the phytosterols on the steroidic excretion was evaluated using gas chromatographic–multiple mass spectrometric ($GC–MS^n$) analyses of the urine samples.

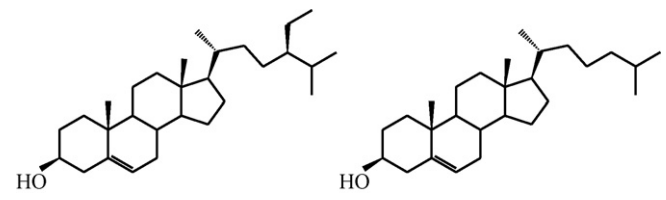

Fig. 2. Chemical structure of the phytosterol β-sitosterol (left) and of cholesterol (right).

2. Materials and methods

2.1. Experimental setup

A small-scale 5-week study was performed with healthy female ($n=6$) and male volunteers ($n=6$), aged 22–48 years old. Volunteers were randomly divided in a control and test group so that both groups consisted of 3 women and 3 men each. It was established that in daily life, these volunteers did not use phytosterol-enriched food products. During the study no restrictions on the normal diet were imposed. But, the control group was asked to keep on refraining from using any phytosterol-enriched food products during the study. The test group was asked to consume a selected commercially available yogurt drink every morning during the first 3 weeks of the study. This drink contained 2 g of phytosterols which is the daily intake recommended by the manufacturer. The last 2 weeks of the study the test group continued their normal diet and refrained from eating or drinking any additional phytosterol-enriched food products.

2.2. Reagents and chemicals

All solvents and reagents were of analytical grade and were purchased from VWR International (Merck, Darmstadt, Germany). β-Zearalanol (3,4,5,6,7,8,9,10,11,12-decahydro-7,14,16-trihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1-one, bZ, purity >98%), boldione (androst-1,4-diene-3,17-dione, ADD, purity ≥98%) and β-testosterone (17β-hydroxy-androst-4-ene-3-one, bT, purity ≥98%) were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥96%), β-boldenone (17β-hydroxy-androsta-1,4-diene-3-one, bBol, purity ≥98%), α-testosterone (17α-hydroxy-androst-4-ene-3-one, aT, purity ≥98%) and equilenin (3-hydroxy-estra-1,3,5,7,9-pentaene-17-one, EQ, purity ≥98%) were purchased from Steraloids (Newport, USA). α-Boldenone (17α-hydroxy-androsta-1,4-diene-3-one, aBol, purity >95%) was provided by The National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Methyl-19-nortestosterone (4-estren-17α-methyl-17β-ol-3-one, MeNT, purity >98%) and androsterone ((3α,5α)-3-hydroxy-androstan-17-one, And, purity ≥98%) were obtained from the Scientific Institute of Public Health (IPH, Brussels, Belgium).

Standard stock solutions were prepared in absolute ethanol at a concentration of 200 ng μL^{-1}. Working standard solutions for spiking were prepared by appropriate dilution of the stock solutions in ethanol. All standard solutions were stored at 4 °C following the quality assurance instructions of Belac accreditation (EN17025).

β-Glucuronidase, Type HP-2, from *Helix pomatia* was purchased from Sigma–Aldrich Corp. and is stored at 4 °C in the laboratory. This preparation contains glucuronidase (≥100 000 units mL^{-1}) as well as sulfatase activity (≤7500 units mL^{-1}).

The derivatisation reagent, $MSTFA^{++}$, was prepared using N-methyl-N-trimethylsilyl-trifluoracetamid (MSTFA, FilterService, Eupen, Belgium), ammonium iodide (Sigma–Aldrich Corp.) and ethanethiol (Acros Organics, Fairlawn, NU, USA) as described by Impens et al. [36].

2.3. Sampling

Volunteers were asked to collect 50 mL urine samples once a day, preferably in the morning before any consumption of drinks or food. Samples were taken at 11 sampling times, one prior to the imposed consumption of phytosterols (period 0) followed by two samples each week during the subsequent 5 weeks. Week

1 through week 3 corresponded to the phytosterol consumption period (period 1), and week 4 through week 5 represented the wash out period (period 2). Immediately after collection, samples were stored at −20 °C until analysis.

2.4. Extraction and clean-up

Extraction was based on the method described by De Brabander et al. [37]. In short, urine pH was adjusted to 4 ± 0.5 before analysis. Analytes were extracted from 25 mL of urine with diethyl ether after enzymatic hydrolysis with β-glucuronidase from *Helix pomatia*. Extracts were dried and resolved in ethanol before filtration and subsequent separation with HPLC. Final analysis was performed with GC–MS–MS after derivatisation with MSTFA^{++}.

2.5. GC–MS–MS analysis

All chromatographic and spectrometric analyses were performed using a Trace Gas Chromatograph 2000 fitted with a Polaris ion trap mass spectrometer (Thermo Fisher, Austin, TX, USA) with a Carlo Erba AS2000 Autosampler (Thermo Fisher). Helium (99.99% purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 mL min^{-1} and perfluorotributylamine (FC43) was used as calibration gas. A sample volume of 1 μL was injected (split flow 60 mL min^{-1}, splitless time 1 min).

Gas chromatographic parameters are based on the method described by Impens et al. [36]. Chromatographic separation of the targeted analytes was performed on a BPX5 capillary column (25 m × 0.22 mm ID) with 5% phenyl-polysilphenylene-siloxane phase (0.25 μm film) (SGE Analytical Science Pty. Ltd., Victoria, Australia). Injector, ion source and transfer line temperature were 250 °C, 200 °C and 275 °C, respectively. The temperature program applied started at an initial temperature of 100 °C. Temperature was increased to 250 °C applying a ramp of 17 °C min^{-1}. Subsequently, an increase to 270 °C was assessed using a ramp of 2 °C min^{-1}. A final ramp of 30 °C min^{-1} was applied to reach 300 °C, holding this temperature for 1.30 min.

Spectra were obtained in positive electron impact ionisation (EI) mode MS full scan and MS–MS scan. Mass range depended on the selected precursor ion, and the collision energy ranged from 0.80 V to 1.20 V.

2.6. Quality assurance

Prior to sample analysis, standard mixtures of the targeted analytes were injected to check the operation conditions of the GC–EI-MS–MS apparatus. Prior to extraction, EQ, MeNT and bZ were added to every sample as procedure internal standards (I.S.) at a concentration of 5.00 ng mL^{-1}. Different internal standards were used so that in each HPLC-fraction one I.S. was present. Different targeted analytes were identified by their retention time, relative to the appropriate I.S., and by the ion ratio of their product ions based on the performance criteria for analytical residue methods as defined in Commission Decision 2002/657/EC [38]. After extraction, And was added to every sample to check the derivatisation efficiency. For quantitative analysis, analyte/internal standard peak area ratios versus spiked concentrations of the analyte were fitted in a calibration curve. Eight point calibration curves were constructed for every single targeted analyte by spiking standard solutions in ultrapure water at a concentration of 0.500–10.0 ng mL^{-1} for ADD, AED, aBol and bBol, and of 0.500–50.0 ng mL^{-1} for aT and bT. For both testosterone isomers (T) extrapolation was allowed for quantification in case this range was exceeded. The limit of quantification (LOQ) was set at the lowest calibration point, 0.500 ng mL^{-1}. Correlation coefficients (R^2) were higher than 0.90 for all targeted analytes.

Since this study entailed the intake of a commercially available yogurt drink at doses recommended by the manufacturer, no authorization from the UGhent Ethics Committee was required to carry out this experiment (as described in Section 2.1).

2.7. Data analysis

Data processing was performed using Xcalibur™ 2.0 software (Thermo Fisher). All data were statistically interpreted using the statistical software package R [39]. To fulfil criteria of normality, concentration data of AED, bT and aT were log transformed. To overcome any problems with missing or zero values, a constant of 1 was added to the concentrations before log transformation. For each analyte, normality of the log transformed data was verified by generating normal quantile–quantile (Q–Q) plots of the residuals.

Correlations between all targeted analytes present in urine (AED, bT, aT and ADD) were assessed computing Spearman's rank correlation coefficients. These correlations were computed for the total dataset, but also for data of the female group, the male group, the control group and the test group separately. Consequently, conclusions at group level could be formulated. The null hypothesis that the Spearman correlation is zero was verified using a permutation test in which permutations were only considered within sample times. All hypothesis tests were performed at the 5% level of significance so that at a *p*-value less than the significance level of 0.05, the null hypothesis was rejected. This procedure is consistent with the design of the study.

To examine any effects of: (1) phytosterol consumption (person-type: control or test persons), (2) different consumption periods (period: period 0, period 1 or period 2) and (3) gender (male or female) on urinary excretion of AED, bT and aT, a linear mixed model with a first order autocorrelation (AR(1)) structure in time and with person as random effect was used. Analysis started with the most complex model one is willing to consider, including the main effects, person-type, period and gender, and their two-factor interactions. The final model was selected using a backward-elimination model selection procedure. Since the main research questions on the influence of phytosterol consumption are related to differences in hormone excretion of control and test persons, and between the three periods, the factors person-type and period were never eliminated from the model. All hypothesis tests were performed at the 5% level of significance using Wald tests. The restricted maximum likelihood (REML) method was chosen for parameter estimation. The most parsimonious models for log AED, log bT and log aT are reported and further discussed.

To statistically interpret data of ADD, their conversion into binary-model-type data, representing either the absence (0) or the presence (1) of ADD in urine, was required. As indicator variable ADD was analysed similarly as log AED, log bT and log aT, except that a generalised linear mixed model was used. This model was further characterised by the binomal distribution, the logit link and also a random effect for person. The Laplace approximation to the likelihood was considered for parameter estimation. Since the logit link is used, the effect sizes are expressed in terms of odds ratios, which are the ratios of the odds of the presence of ADD in one group (e.g. gender, period, etc.) as compared to the odds in another group. The odds of the presence of ADD is defined as the probability that ADD is present divided by the probability that ADD is absent. All hypothesis tests were performed at the 5% level of significance using Wald tests.

3. Results

Urine samples were screened for aBol, bBol, ADD, AED, bT and aT. As presented in Table 1, boldenone (Bol) was not detected in any of

Table 1
Urinary concentrations of ADD, AED, bT and aT (mean ± SD) for female and male control and test persons in period 0, period 1 and period 2.

		Female			Male		
		Period 0	Period 1	Period 2	Period 0	Period 1	Period 2
ADD (ng mL^{-1})	Control persons	nd[a] (0/2)[b]	nd (0/17)	nd (0/12)	1.33 (1/2)	nd (0/17)	nd (0/11)
	Test persons	nd (0/1)	nd (0/17)	0.909 (1/12)	0.769 ± 0.0254 (2/3)	1.34 ± 0.557 (2/18)	nd (0/11)
AED (ng mL^{-1})	Control persons	1.28 ± 1.00 (2/2)	5.69 ± 5.50 (15/17)	6.81 ± 5.34 (12/12)	1.35 (1/1)	5.87 ± 3.94 (17/17)	7.96 ± 4.31 (11/11)
	Test persons	0.729 (1/2)	5.05 ± 4.62 (15/17)	12.4 ± 22.9 (11/12)	1.30 ± 0.235 (3/3)	2.77 ± 1.72 (17/18)	9.79 ± 14.0 (10/11)
bT (ng mL^{-1})	Control persons	1.67 ± 0.175 (2/2)	2.14 ± 0.328 (7/17)	1.91 ± 0.463 (7/12)	13.7 (1/1)	12.1 ± 10.1 (16/17)	14.4 ± 20.4 (10/11)
	Test persons	3.02 ± 0.941 (2/2)	3.96 ± 2.63 (14/17)	2.47 ± 0.855 (10/12)	2.86 ± 2.11 (3/3)	10.1 ± 6.83 (16/18)	10.7 ± 11.6 (11/11)
aT (ng mL^{-1})	Control persons	1.71 ± 0.244 (2/2)	4.52 ± 4.88 (14/17)	3.65 ± 2.10 (11/12)	36.8 (1/1)	40.7 ± 44.9 (16/17)	74.7 ± 108 (10/11)
	Test persons	3.70 ± 1.39 (2/2)	5.33 ± 4.33 (16/17)	3.17 ± 1.66 (12/12)	3.53 ± 2.74 (2/3)	10.3 ± 7.51 (15/18)	12.0 ± 12.0 (11/11)

[a] nd, not detected.
[b] (x/n) with x = number of samples in which the analyte was detected, n = total number of samples analysed over that period.

the urine samples. The presence of boldione (ADD), the direct precursor of Bol, was demonstrated sporadically in period 0, 1 and 2 in some male and female volunteers of both the control and test group (Table 1). In Fig. 3 chromatogram and spectrum of ADD detected in urine of a male volunteer are compared with those of a standard solution of this analyte. ADD could be detected in urine samples in concentrations ranging from 0.751 ng mL^{-1} to 1.73 ng mL^{-1}. It should be noted that the detected ADD concentrations were close to the limit of quantification (0.500 ng mL^{-1}). All other targeted analytes were frequently present in the urine of both male and female volunteers (Table 1).

Table 2 presents the correlations between the urinary analytes established using the non-parametric Spearman's rho correlation coefficient. A positive correlation indicates a tendency that both variables decrease or increase simultaneously, whereas a negative correlation indicates that when one variable increases, the other tends to decrease, and vice versa.

Between bT and its precursor AED, a significantly positive correlation could be established (p = 0.0006) and this correlation could be totally attributed to men (p_{male} = 0.0000 versus p_{female} = 0.2580). Also aT was significantly positively correlated to AED (p = 0.0000). But this correlation was not gender dependent, although it was stronger in male than in female volunteers (p_{male} = 0.0000 versus p_{female} = 0.0080). bT and aT appeared to be positively correlated (p = 0.0000). In contrast, the significant negative correlation found between ADD and aT (p = 0.0066) could be only attributed to male volunteers (p_{male} = 0.0100 versus p_{female} = 0.3340). A slightly negative but non-significant relation could be established between ADD and AED and between ADD and bT. Moreover, these correlations were stronger in male than in female volunteers (ADD–AED: p_{male} = 0.3380 versus p_{female} = 1.0220, and ADD–bT: p_{male} = 0.01600 versus p_{female} = 1.0400).

No influence of phytosterol consumption could be attributed to the observed positive correlations between bT and AED, aT and AED and bT and aT ($p_{control}$ = 0.0000 versus p_{test} = 0.0580, $p_{control}$ = 0.0020 versus p_{test} = 0.0080, and $p_{control}$ = 0.0000 versus p_{test} = 0.0000, respectively). Remarkably, all correlations in which ADD is considered, were stronger in the test group than in the control group (ADD–AED: $p_{control}$ = 0.9780 versus p_{test} = 0.1540, ADD–bT: $p_{control}$ = 0.9480 versus p_{test} = 0.3260, and ADD–aT: $p_{control}$ = 0.9400 versus p_{test} = 0.0300). Based on these results, it cannot be excluded that the latter correlations experienced an influence of phytosterol consumption.

No statistically significant effect of phytosterol consumption on the excretion of the anabolic steroids could be established.

The effect of period on the mean log AED, however, was found significant (p = 0.0034), but, this was true for both the control and the test group. No significant effect of person-type on the mean log AED could be established (p = 0.3555). Pair-wise comparisons of the mean log AED between the three periods revealed that the mean log AED in period 2 proved to be 0.744 larger than in period 0 (95% CI: [0.224, 1.26], p = 0.0050) and 0.368 larger than in period 1 (95% CI: [0.107, 0.629], p = 0.0058). However, the mean difference in log AED between periods 1 and 0, was not significant. This difference was estimated to be 0.376 (95% CI: [−0.130, 0.882], p = 0.1457).

The model selection for log bT resulted in a model with a significant gender effect (p = 0.0003) but a non-significant effect of period (p = 0.9045) and person-type (p = 0.3254). The mean difference in log bT between males and females was estimated to be 1.19 (95% CI: [0.780, 1.60]).

Log aT also exhibited a significant effect of gender (p = 0.0012) and of the interaction of gender and person-type (p = 0.0157). The effect of period and of person-type on the mean log aT was not significant (respectively, p = 0.3419 and p = 0.0598). The mean log aT for males in the control group was 1.93 (95% CI: [1.26, 2.61]) larger than for females in the control group. In the test group the mean difference was estimated to be as low as 0.454 (95% CI: [−0.213, 1.12]). For males, the mean log aT in the test group was expected to be 1.29 (95% CI: [−1.96, −0.62]) smaller than in the control group. For females, the mean difference in log aT between test and control persons was estimated to be 0.188 (95% CI: [−0.482, 0.858]).

The presence of ADD was significantly affected by the period (p = 0.0060). In particular, the odds ratio of the presence of ADD in period 2 as compared to period 0 was estimated to amount 0.0219 (95% CI: [0.00124, 0.393], p = 0.0094). For period 1 as compared to period 0, the estimated odds ratio equalled 0.0282 (95% CI: [0.00266, 0.298], p = 0.0030). The presence of ADD in period 2 in comparison with period 1 was not significant (odds ratio equalled 0.782, p = 0.8651). Also the effect of person-type on the presence of ADD was not significant (p = 0.1324).

Table 2
Spearman's rank correlation coefficients for the total dataset between concentrations of AED, bT, aT and ADD detected in urine.

	AED	bT	aT	ADD
AED	1.00 (n = 132)	0.329[a] (n = 132)	0.471[a] (n = 132)	−0.110 (n = 132)
bT	0.329[a] (n = 132)	1.00 (n = 132)	0.800[a] (n = 132)	−0.218 (n = 132)
vaT	0.471[a] (n = 132)	0.800[a] (n = 132)	1.00 (n = 132)	−0.288[a] (n = 132)
ADD	−0.110 (n = 132)	−0.218 (n = 132)	−0.288[a] (n = 132)	1.00 (n = 132)

[a] Correlation is significant at the 5% significance level.

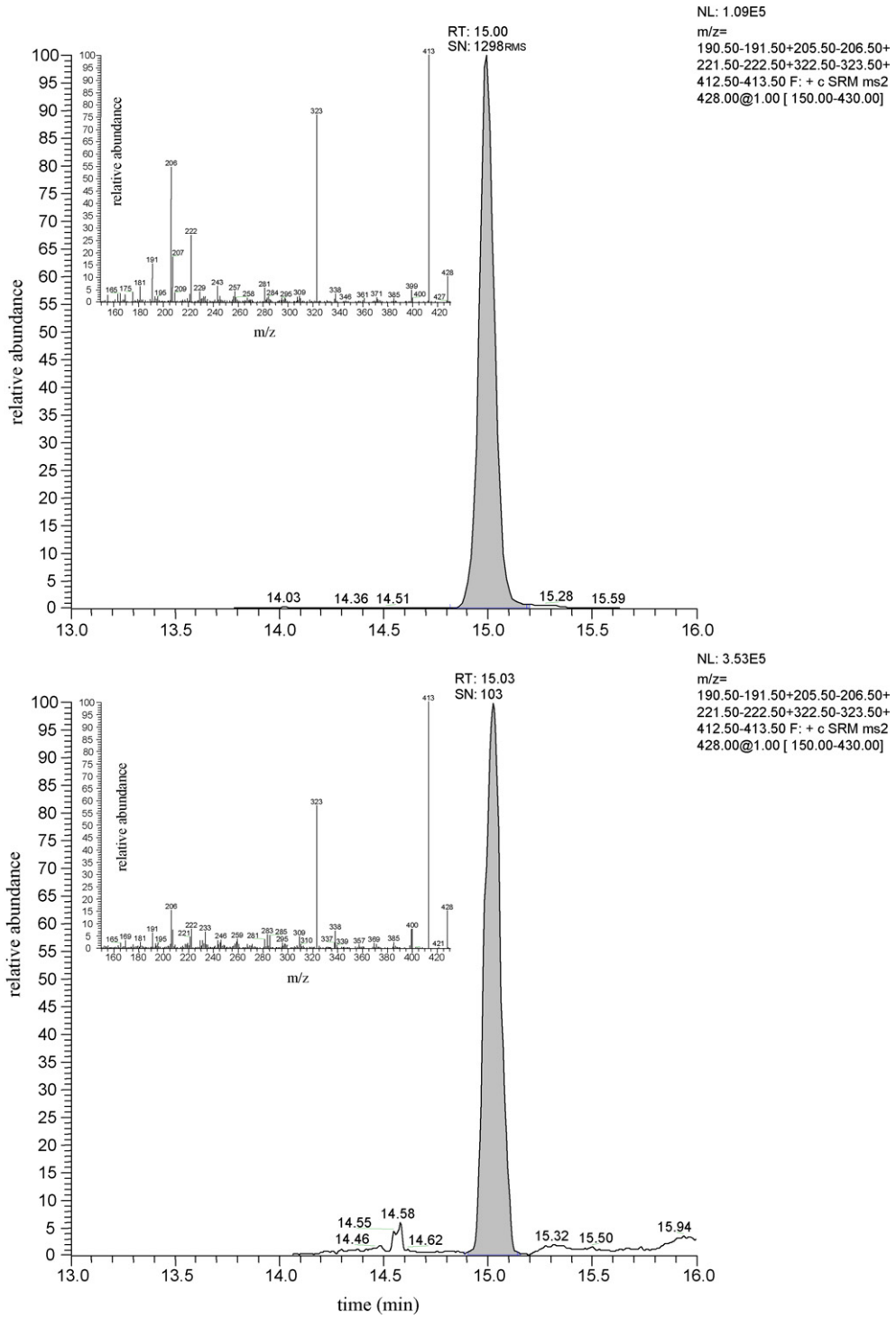

Fig. 3. Chromatogram and spectrum (inset) of boldione (ADD): (up) standard solution (2.00 ng on column) and (under) urine sample of a male test person (1.33 ng on column).

4. Discussion

In this human *in vivo* trial ADD, the direct precursor of Bol, was sporadically detected in urine samples of both male and female volunteers. The detection of this anabolic steroid in the control group as well as in the test group who had consumed phytosterols points to its endogenous origin. Influences of oral phytosterol consumption on this natural production and consequently the urinary excretion of ADD and other anabolic steroids could not be univocally demonstrated.

The most striking observation of the present study was the occurrence of ADD positive urine samples in the control group. For doping control agencies this finding may be of considerable importance because ADD is still listed as a prohibited exogenous anabolic steroid [2]. However, a comment has been included in this list indicating that in rare cases the production of these anabolic steroids listed as exogenous, may be endogenous. In case of a positive urine sample, microbial activity after sampling has frequently been mentioned as the cause of false positive results [10,40,41]. In our study however, special care has been taken not

to contaminate the samples with gastro-intestinal flora during collection. Moreover, degradation of the samples owing to inaccurate storage conditions was prevented by immediately storing the samples at −20 °C after collection.

As studied by Kim et al. [3], administration of ADD leads to Bol in humans. In their study, the urinary excretion profile of a healthy male volunteer was evaluated over 48 h after oral administration of one tablet of ADD (100 mg tablet^{-1}). Bol was the main metabolite excreted in urine. From their experiment it was also concluded that the excreted amount of Bol reached its maximum (971 ng mL^{-1}) 3.6 h after administration of ADD and decreased to 486 ng mL^{-1} after 48 h. However, the exact pathway describing human ADD metabolism to Bol has not been elucidated and no long-term data on Bol excretion by humans are available.

Since ADD, the direct precursor of Bol, was occasionally detected in urine in this study, detection of Bol was also expected. However, compared to an administered ADD concentration of 100 mg [3], naturally occurring concentrations of this analyte might have been too low to allow for Bol formation. Sub-optimal enzymatic conditions in the human intestinal tract of our subjects might also explain the lack of Bol production. The importance of the environmental conditions for ADD and Bol incidence has been discussed by Mareck et al. [40]. In this review, a study was reported in which the human gastro-intestinal tract was simulated to establish optimal conditions for the formation of ADD, Bol and its metabolites. In simulations with enhanced oxygen conditions, the increased redox-potential in the gut led to large quantities of ADD while Bol could not be detected. This observation may explain the occasional detection of ADD as established in our study.

Next to Bol and ADD, the urinary excretion of AED and of bT and aT, two stereoisomers of testosterone (T), was also measured in our study. These anabolic steroids are relevant for human steroidogenesis and are metabolically linked. For this reason, specific interactions between these analytes were investigated. Between AED and both T isomers a positive correlation was established, which can be explained by the fact that AED is the direct precursor of T [12,18]. An increase in AED will therefore lead to increased T production and excretion. ADD is a metabolite of T [9], and this is reflected in our study by the negative correlation between both T stereoisomers and ADD. Metabolism of T, and thus a decrease in T concentration, will result in an increase of its metabolite ADD. AED can also be considered an important metabolite of T [9]. However, a positive correlation between AED and T was observed in our study. This may be explained by AED being of higher importance as precursor of T than as its metabolite. Not unexpectedly, all correlations described here were more obvious in the male than in the female volunteers and some can be totally attributed to males. An explanation for these results may be that all anabolic steroids under consideration were male sex hormones [1,7].

Urinary excretion of hydroxylated metabolites of T generated by different isoenzymes of cytochrome P450, such as AED and ADD, appears to be relatively low [7]. Although ADD concentrations were close to the limit of detection, in our study bT, aT, and AED could be detected at concentrations well above their limits of detection using GC–MS–MS. Since 1972 mass spectrometry (MS) is used to analyse urine samples for doping control purposes [4]. Combined with gas or liquid chromatography (GC or LC) and various ionisation methods, mass spectrometry enables reliable screening and confirmation of anabolic steroids in different matrices. Up till now, its specificity and sensitivity have made GC–MS^n the method of choice for detection of anabolic steroids in urine [4,42,43]. However, mainly due to reduced sample pre-treatment, particularly by the lack of derivatising, LC–MS–MS is gradually becoming more important for doping analyses purposes [5].

Within this research framework, it was attempted to relate the urinary excretion of anabolic steroids to phytosterol consumption. The success of phytosterols as functional-food ingredients is related to their cholesterol-lowering activity. The competition of phytosterols and cholesterol for absorption from the gastro-intestinal tract has been described extensively [13,14,16,17]. Moreover, it has been demonstrated that absorption of phytosterols is limited compared to that of cholesterol [15–17]. Consequently, phytosterols spend a longer time in the gastro-intestinal tract, possibly allowing their microbial conversion into androgenic products [8]. Therefore it was assumed that short-term increased intake of phytosterols by humans could alter their urinary excretion of anabolic steroids. Based on our 5-week *in vivo* trial, no significant effect of phytosterol consumption on anabolic steroids' excretion could be demonstrated. More particularly, no relation between the incidence of ADD and phytosterol consumption could be established. ADD was, however, more related to other anabolic steroids following phytosterol consumption. The lack of a significant influence of phytosterols on ADD excretion is inconsistent with reports on the microbial transformation of phytosterols into ADD [44–46]. These *in vitro* experiments demonstrated that ADD production was positively correlated to the presence of phytosterols. Although *in vitro* experiments can give good indications, the present comparison indicates that this approach does not always reflect what may occur in the *in vivo* situation. Previous *in vivo* research conducted by Ros et al. [35] corroborates the results of our study. It must be emphasised that in both studies phytosterols were consumed at the doses recommended by the manufacturers.

Considerable individual variation in concentrations of urinary steroids' excretion was observed in our study (Table 1). However, the wide intra- and inter-individual variability in excreted testosterone concentrations has been previously demonstrated by several studies [10,47,48]. To accurately perform steroid profiling, the ratio of testosterone (T) to epitestosterone (E) should have been investigated [10]. Under natural circumstances, the endogenous T/E ratio is not intra-individually variable but remains constant [6]. Although this ratio is sensitive to variations between individuals, inter-individual comparison of this ratio would probably be more reliable than comparing absolute steroid concentrations. However, within the scope of our study, especially for analysis of ADD and Bol, steroid profiling was not specifically required. But it might be of particular interest for any long-term evaluation of the potential effects of phytosterol consumption on human steroidogenesis.

5. Conclusion

Particularly prominent in this study was the detection of endogenous boldione or ADD, the direct precursor of boldenone, in human urine using GC–MS–MS. Urinary boldenone excretion could not be proven, whereas other anabolic steroids such as AED, aT and bT were frequently excreted by both males and females. No evidence of phytosterol related anabolic steroids' excretion was observed after consumption of phytosterol containing functional food at the recommended dose. Considering the relatively short-term duration of this *in vivo* trial and the small sample size, it is recommended to conduct more extensive long-term studies to further explore the potential interferences of phytosterols with human steroidogenesis and excretory steroid patterns.

Acknowledgements

This study was financially supported by the Special Research Fund (BOF). The authors wish to acknowledge all volunteers for their efforts during the trial. Also thanks to A. Van Bastelaere for her contribution to the trial performance and all technical staff for their practical assistance in the laboratory. Finally, D. Stockx is acknowledged for his contribution to the development of the GC–MS–MS method.

References

[1] A.T. Kicman, Review: pharmacology of anabolic steroids, Br. J. Pharmacol. 154 (2008) 502–521.
[2] WADA, The World Anti-Doping Agency, The 2008 Prohibited List (last consulted 17/03/2009), http://www.wada-ama.org, 2008.
[3] Y. Kim, M. Jun, W. Lee, Characterisation of boldione and its metabolites in human urine by liquid chromatography/electrospray ionisation mass spectrometry and gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 9–20.
[4] M. Thevis, W. Schänzer, Mass spectrometry in doping control analysis, Curr. Org. Chem. 9 (2005) 825–848.
[5] O.J. Pozo, P. Van Eenoo, K. Deventer, F.T. Delbeke, Detection and characterisation of anabolic steroids in doping analysis by LC–MS, Trends Anal. Chem. 27 (8) (2008) 657–671.
[6] D.H. van de Kerkhof, Endogene steroïden: een aparte tak van sport bij doping-analyse, Ned. Tijdschr. Klin. Chem. Labgeneesk. 31 (1) (2006) 41–46.
[7] W. Schänzer, Metabolism of anabolic androgenic steroids, Clin. Chem. 42 (7) (1996) 1001–1020.
[8] H.F. De Brabander, K. Verheyden, V. Mortier, B. Le Bizec, W. Verbeke, D. Courtheyn, H. Noppe, Phytosterols and anabolic agents versus designer drugs, Anal. Chim. Acta 586 (2007) 49–56.
[9] I.M. Bird, A.J. Conley, Steroid biosynthesis: enzymology, integration and control, in: J.I. Mason (Ed.), Genetics of Steroid Biosynthesis and Function, Routledge, USA, 2003, p. 479.
[10] A.T. Kicman, D.B. Gower, Anabolic steroids in sport: biochemical, clinical and analytical perspectives: a review, Clin. Biochem. Rev. 40 (2003) 321–356.
[11] I.A. Macdonald, V.D. Bokkenheuser, J. Winter, A.M. McLernon, E.H. Mosbach, Degradation of steroids in the human gut, J. Lipid Res. 24 (1983) 675–700.
[12] A.H. Payne, D.B. Hales, Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones, Endocr. rev. 25 (2004) 947–970.
[13] W.H. Ling, P.J.H. Jones, Minireview: dietary phytosterols: a review of metabolism, benefits and side effects, Life Sci. 57 (1995) 195–206.
[14] V. Piironen, D.G. Lindsay, T.A. Miettinen, J. Toivo, A.-M. Lampi, Review: plant sterols: biosynthesis, biological function and their importance to human nutrition, J. Sci. Food Agric. 80 (2000) 939–966.
[15] S. Rozner, N. Garti, The activity and absorption relationship of cholesterol and phytosterols, Colloids Surf. A: Physicochem. Eng. Aspects 282–283 (2006) 435–456.
[16] A. de Jong, J. Plat, R.P. Mensink, Metabolic effects of plant sterols and stanols (Review), J. Nutr. Biochem. 14 (2003) 362–369.
[17] T. Heinemann, G. Axtmann, K. Van Bergmann, Comparison of intestional absorption of cholesterol with different plant sterols in man, Eur. J. Clin. Invest. 23 (1993) 827–831.
[18] S. Fritsche, H. Steinhart, Occurrence of hormonally active compounds in food: a review, Eur. Food Res. Technol. 209 (1999) 153–179.
[19] K.B. Hicks, R.A. Moreau, Phytosterols and phytostanols: functional food cholesterol busters, Food Technol. 55 (2001) 63–67.
[20] P. Laakso, Analysis of sterols from various food matrices, Eur. J. Lipid Sci. Technol. 107 (2005) 402–410.
[21] W. Verbeke, Consumer acceptance of functional foods: socio-demographic, cognitive and attitudinal determinants, Food Qual. Prefer. 16 (2005) 45–57.
[22] S.F.L. Kirk, D. Greenwood, J.E. Cade, A.D. Pearman, Public perception of a range of potential food risks in the United Kingdom, Appetite 38 (2002) 189–197.
[23] M. Tucker, S.R. Whaley, J.S. Sharp, Consumer perceptions of food-related risks, Int. J. Food Sci. Technol. 41 (2006) 135–146.
[24] W. Verbeke, L.J. Frewer, J. Scholderer, H.F. De Brabander, Why consumers behave as they do with respect to food safety and risk information, Anal. Chim. Acta 586 (2007) 2–7.
[25] O.V. Egorova, S.A. Gulevskaya, I.F. Puntuts, A.E. Filonov, M.V. Donova, Production of androstenedione using mutants of *Mycobacterium sp*, J. Chem. Technol. Biotechnol. 77 (2002) 141–147.
[26] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.M.S. Cabral, Microbial conversion of steroid compounds: recent developments, Enzyme Microb. Technol. 32 (2003) 688–705.
[27] C.-L. Huang, Y.-R. Chen, W.-H. Liu, Production of androstenones from phytosterol by mutants of *Mycobacterium sp*, Enzyme Microb. Technol. 39 (2006) 296–300.
[28] K. Kieslich, Review: microbial side-chain degradation of sterols, J. Basic Microb. 25 (1985) 461–474.
[29] C.-K. Lo, C.-P. Pan, W.-H. Lu, Production of testosterone from phytosterol using a single-step microbial transformation by a mutant of *Mycobacterium sp*, J. Ind. Microbiol. Biotechnol. 28 (2002) 280–283.
[30] S.B. Mahato, S. Garai, Review: advances in microbial steroid biotransformation, Steroids 62 (1997) 332–345.
[31] A. Malaviya, J. Gomes, Androstenedione production by biotransformation of phytosterols, Bioresour. Technol. 99 (2008) 6725–6737.
[32] I. Christanson-Heiska, P. Smeds, N. Granholm, E. Bergelin, B. Isomaa, Endocrine modulating actions of a phytosterol mixture and its oxidation products in zebrafish (*Danio rerio*), Comp. Biochem. Physiol. 145 (2007) 518–527.
[33] S. Poelmans, K. De Wasch, Y. Martelé, R. Schilt, N. Van Hoof, H. Noppe, T. Verslycke, C.R. Janssen, D. Courtheyn, H.F. De Brabander, The possible transformation of phytsosterols to boldenone, in: Proceedings Euro Food Chem XII: Strategies for Safe Food 24–26/09/2003, Bruges, Belgium, 2003, pp. 74–78.
[34] K. Verheyden, H. Noppe, V. Mortier, J. Vercruysse, E. Claerebout, P. Van Immerseel, C.R. Janssen, H.F. De Brabander, Formation of boldenone and boldenone-analogues by maggots of *Lucilia sericata*, Anal. Chim. Acta 586 (2007) 163–170.
[35] M.M. Ros, S.S. Sterk, H. Verhagen, A.F.H. Stalenhoef, N. de Jong, Phytosterol consumption and the anabolic steroid boldenone in humans: a hypothesis piloted, Food Addit. Contam. 24 (7) (2007) 679–684.
[36] S. Impens, K. De Wasch, M. Cornelis, H.F. De Brabander, Analysis on residues of estrogens, gestagens and androgens in kidney fat and meat with gas chromatography–tandem mass spectrometry, J. Chromatogr. A 970 (2002) 235–247.
[37] H.F. De Brabander, P. Vanhee, S. Van Hoye, R. Verbeke, Improved chromatographic clean-up of anabolics in bovine urine, J. Planar Chromatogr. 2 (1989) 33–38.
[38] Office for Official Publications of the European Communities, Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results, L221, 2002, 8–36.
[39] R Development Core Team, R: a language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2008, ISBN 3-900051-07-0 (last consulted 18/03/2009) http://www.R-project.org.
[40] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Schänzer, Review: factors influencing the steroid profile in doping control analysis, J. Mass Spectrom. 43 (2008) 877–891.
[41] World Anti-Doping Agency: WADA Technical Document – TD2004 EAAS, Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids, 2004, (last consulted 18/03/2009) http://www.wada-ama.org/rtecontent/document/end_steroids_aug_04.pdf.
[42] S. Impens, J. Van Loco, J.M. Degroodt, H. De Brabander, A downscaled multi-residue strategy for detection of anabolic steroids in bovine urine using gas chromatography tandem mass spectrometry (GC–MS^3), Anal. Chim. Acta 586 (2007) 43–48.
[43] M.N. Saugy, C. Schweizer, Test methods: anabolics, Baillière's Clin. Endocrinol. Metabol. 14 (2000) 111–133.
[44] K. Sarangthem, T.N. Singh, Biosynthesis of succulent bamboo shoots of *Bambusa balcooa* into phytosterols and its biotransformation into ADD, Acta Bot. Sin. 45 (2003) 114–117.
[45] K. Sarangthem, T.N. Singh, Microbial bioconversion of metabolites from fermented succulent bamboo shoots into phytosterols, Curr. Sci. 84 (2003) 1544–1547.
[46] Z. Wang, F. Zhao, D. Chen, D. Li, Biotransformation of phytosterol to produce androsta-diene-dione by resting cells of *Mycobacterium* in cloud point system, Process Biochem. 41 (2006) 557–561.
[47] E. Raynaud, M. Audran, J.C. Pages, J.F. Brun, C. Fedou, J.L. Chanal, A. Orsetti, Urinary excretion of testosterone and epitestosterone glucuronides in preadolescents and adolescents, Pathol. Biol. 41 (1993) 159–163.
[48] E. Raynaud, M. Audran, J.C. Pages, C. Fedou, J.F. Brun, J.L. Chanal, A. Orsetti, Determination of urinary testosterone and epitestosterone during pubertal development. A cross-sectional study in 141 normal male subjects, Clin. Endocrinol. 38 (1993) 353–359.