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Phytosterol consumption and the anabolic steroid boldenone in humans: a hypothesis piloted

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Abstract

The presence of the anabolic steroid boldenone in animals has become a research topic as its occurrence is proposed to be a marker for illegal hormone administration. However, boldenone can also be formed from β–sitosterol, a phytosterol present in animal feed, as well as from endogenous sources. The observations in animals together with the increased consumption of phytosterol-enriched foods in the western population led us to the hypothesis that consumption of phytosterol-enriched foods might possibly lead to increased boldenone levels in humans. We performed a pilot study among female volunteers (n=10) to investigate whether boldenone concentrations in urine were detectable after consumption of 25 g/day of phytosterol-enriched margarines for one week. Urine samples were collected at day 0, day 3 or 4 and day 7. Urine of a sitosterolemia (a rare autosomal recessively inherited lipid metabolic disorder) patient was collected as a positive control case. No traces of boldenone were detected in either the volunteers or in the patient. In conclusion, there is no evidence of formation of boldenone in women after consumption of the recommended amount of phytosterol-enriched margarines.

Introduction

For a number of years, the presence of the anabolic steroid boldenone (1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one) in various animal species has become a topic of research as the occurrence of this hormone or its metabolites in biological samples is proposed to be a marker for illegal hormone administration (De Brabander and others 2004). Especially urine samples of cattle and veal calves have been subject of research in various EU countries. The investigation into the origin of the boldenone in bovine urine is still ongoing. Boldenone can be formed from β – sitosterol (a phytosterol naturally occurring in plants) present in animal feed, but also endogenous production can still be one of the sources (Poelmans and others 2005). Phytosterols are a normal constituent of the human diet. Normal levels of consumption are approximately 200-400 mg/day, which may increase to 700-800 mg/day in those who consume a large amount of soy-based foods (Andersson and others 2004). The recently introduced phytosterol-enriched foods deliver 2-3 g of phytosterols per day if the recommended amount is consumed. The intestinal absorption of phytosterols is estimated between 0.4-3.5% (Hallikainen and others 2000).

The observations in animals together with the increased consumption of phytosterol-enriched foods in the western population to lower (slightly) elevated serum cholesterol levels lead us to the hypothesis that in humans phytosterol-enriched food may possibly lead to an unwanted amount of the anabolic steroid boldenone as in animals. This would then identify a new potential health hazard in humans.

Phytosterol-enriched margarines are on the market for some years now, and other plant sterol-enriched food products including dairy products have also been introduced. The

phytosterols are thought to displace cholesterol from mixed micelles in the intestine and thereby reduce intestinal cholesterol absorption; the exact mechanism, however, remains still unknown (Katan and others 2003). In animals boldenone works as an anabolic agent and is misused in cattle fattening. It is commonly used to enhance athletic performance and muscular development in humans. Hypertension, heart attacks, strokes, liver and other types of internal organ cancers have been associated with anabolic steroid use (De Brabander and others 2004; Sullivan and others 1998).

Within the framework of Postlaunch Monitoring (PLM) of functional foods in which positive but also potential side effects are investigated (de Jong and others 2004; 2005a; 2005b; Wolfs and others 2006) we studied the hypothesis postulated above. To our knowledge there were no data of biotransformation of the phytosterol β-sitosterol into boldenone in humans. We performed a pilot study in 10 women to investigate whether boldenone concentrations in urine were detectable after consumption of the recommended daily amounts of phytosterol-enriched margarines. In addition, a urine sample of a sitosterolemia patient was analysed as a positive control case. Patients with sitosterolemia are characterized by a > 50-fold elevation in plasma phytosterol levels, which results from an increased absorption and decreased hepatic removal of phytosterols that leads to accumulation of phytosterols in blood and tissues (Ketomaki and others 2005). Because these patients have very high levels of plasma phytosterols we anticipated that especially in these patients the presence of boldenone might be demonstrated.

Materials and methods

Study design

For our pilot 10 healthy female volunteers (aged 22 to 51) were recruited. In daily life, these women did not use phytosterol-enriched margarines. We included only women because they excrete less to none anabolic hormones by nature. During our one week trial we provided phytosterol-enriched margarines. The participants were asked to consume 25 grams per day (i.e. 2 grams of phytosterols or ~1 gram of β-sitosterol) of the phytosterol-enriched margarines, which corresponds to the recommended intake by the manufacturer. At the beginning of the study 7 portions of 25 grams were distributed to the volunteers. Spot urine samples were collected on day 0, on day 3 or 4, and on day 7 at around lunchtime. In this way we collected one voidance from a day at about the same time. Within 30 minutes after collection the urine samples were frozen at minus 20^{o}C. As a normal food product was supplied in physiological quantities and the collection of spot urine samples was not invasive, no authorization from the medical ethical committee was necessary (personal communication with the Medical Committee of Ethics of the University Medical Centre Utrecht, April 2006). The volunteers received a little incentive after participation.

In the blind urine samples, ß-sitosterol, campesterol, stigmasterol, ß-boldenone, α-boldenone, α-testosterone and ß-testosterone were analysed according to ARO-SOP 507 (ARO-SOP 507 RIVM: Bilthoven) a detailed description of which follows below. In addition, one spot urine sample of our positive control case, i.e. a patient with sitosterolemia, was collected and analysed.

Method of analysis

Chemicals

All chemicals and reagents were of high purity quality. α/ß-boldenone, α/ß-testosterone, androsterone, etiocholanolon, α-androsterone, testosterone-D2 and ß-boldenone-D3 were obtained from the RIVM-CRL, Bank of Reference Standards. Cholesterol, coprostanol, campesterol, ß-sitosterol, stigmasterol were obtained from Matreya. Cholesterol-D6 was obtained from Isotel. ß-glucuronidase from E.Coli K12 (Roche). Derivatization reagent for coprostanol, cholesterol, cholesterol-D6, campesterol and sitosterol consists of 25 µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) / ammoniumiodide/dithiothreitol (1000:2:4, v/w/w) (Alltech), for α/ß-boldenone, α/ß-testosterone, androsterone, etiocholanolon, α-androsteron, testosterone-D2 and ß-boldenone-D3 the derivatization reagent consisted of 10 µl heptafluorobutyric Acid Anhydride (HFAA) (Pierce) and 40 µl of dried acetone. Phosphate buffer pH 7.4 was prepared by dissolving 2.278 g of disodiumhydrogenphosphate and 0.416 g of potassium-dihydrogenphosphate in 800 ml of water, the pH was adjusted to 7.4 ± 0.1 and water was added to a final volume of 1000 ml.

Apparatus

Liquid Chromatography (LC): Waters Chromatography autosampler, two Waters pumps, Pharmacia controller, ThermoQuest multi-channel UV-detector. HPLC-column used was a Superspher RP-18 (L 125 mm, 4 mm ID, 4 µm) with pre-column (Waters). Column temperature 40°C. Fraction-collector (Foxy Jr). Datasystem, PC1000 ThermoQuest. The LC mobile phase consists of solution A: 65:35 v/v-% methanol/water, solution B: 100% methanol. Gradient starts at 0% B, after 8 min the percentage B is increased in 8 min to 100% and remains at 100% till 25 min. The gradient then returns in 0.1 min to the initial condition. The flow rate was 0.7 ml/min.

Gas-Chromatography coupled to a mass-spectrometer (GC-MS) analysis was carried out on an Agilent 5973 MSD. GC capillary column, 30 meter VF-17MS (Varian) i.d. 0.25 mm, 0.15 µm film thickness, constant flow of 1.1 ml helium/minute. Injection, splitless mode at 250°C, injection volume 2 µl. The oven temperature was kept constant at 80°C for 1 min and was increased, 20°C per min, to 340°C and was kept constant at this temperature for 4 min.

Extraction procedure

Isolation

A sample portion of 5 ml of urine was transferred to a 10 ml glass tube. The samples were spiked with 10 ng ß-boldenone-D3 , 10 ng testosterone-D2 and 25 cholesterol-D6. To the samples 1 ml of phosphate buffer (pH 7.4) and 50 µl of ß-glucuronidase was added. The mixture was vortexed and hydrolysed for 3 hours at 52°C.

A SPE C_{18} column (3 ml) was preconditioned with 3 ml of methanol and 3 ml of water. The centrifuged sample was passed through the column. The SPE C_{18} column was washed with 3 ml of water and 3 ml 30:70 v/v-% acetonitrile/water. The anabolic steroids were eluted with 4 ml of 80:20 v/v-% methanol/water. The eluate was evaporated at 55°C under a gentle stream of nitrogen until dry and further processed as described in Preparative HPLC. The sterols were eluted with 3 ml of iso-octane, the eluate was collected and evaporated at 55°C under a gentle nitrogen until dryness and derivatised as described under derivatisation.

Preparative HPLC

The dried extracts were reconstituted in 120 µl of 65:35 v/v-% methanol/water, from which 100 µl was injected. One fraction was collected from 4.9 to 20 minutes. The collected fraction was dried under a stream of nitrogen. The dried extracts were reconstituted in 300 µl ethanol, transferred to a 2 ml vial and evaporated at 55°C under a gentle stream of nitrogen to dryness.

Derivatisation

The dried extracts containing cholesterol, coprostanol, campesterol, sitosterol and cholesterol-D6 were reconstituted in 20 µl of derivatisation reagent (MSTFA++) and incubated for one hour at 60°C. After 1 hour the derivatisation reagent was evaporated. The dried residue was reconstituted in 75 µl of iso-octane.

The dried extracts containing α/ß-boldenone, α/ß-testosterone, α/ß-androsterone, etiocholanolon, testosterone-D2 and ß-boldenone-D3 were reconstituted in 50 µl of derivatisation reagent (HFBA/aceton) and incubated for one hour at 60°C. After 1 hour the derivatisation reagent was evaporated. The dried residue was reconstituted in 50 µl of iso-octane.

Detection

Mass spectrometric detection was performed in selected ion monitoring (SIM) mode, see Table 1 and 2 for an overview of the m/z monitored and typical retention times of the analytes.

Results

In Table 3 the results of our pilot study are presented. Unexpectedly and in contrast with our hypothesis no traces of boldenone in the urine of any of the volunteers could be detected. The levels of ß-sitosterol, campesterol and stigmasterol were almost the same over the 7 days of consumption of phytosterol enriched margarines. Also the patient with sitosterolemia showed no traces of urinary boldenone. As expected, the patient levels of ß-sitosterol, campesterol and stigmasterol were elevated compared to the mean levels of the female volunteers. Table 4 presents prelimary data on urinary levels of α-testosterone and ß-testosterone, which showed a slight increase in testosterone compared to baseline levels, after a few days of phytosterol-enriched margarine consumption.

Discussion

From our pilot we conclude that there is no evidence of formation of the anabolic steroid boldenone in women volunteers after consumption of the recommended amount (i.e. 25 grams per day) of phytosterol-enriched margarines. In addition, although the urinary sitosterol and stigmasterol levels in our sitosterolemia patient are elevated as expected, also no urinary boldenone in this positive control case could be detected. Therefore, within the PLM perspective of this study there seems no health hazard for women forming boldenone associated with this type of functional food.

In contrast to the clear results on the absence of urinary boldenone in both our human volunteers and in our sitosterolemia patient, the results on urinary testosterone are rather uncertain. At day 3 or 4 the ratio of β-testosterone and α-testosterone seems to be high, but this value was influenced by one low concentration of α-testosterone near the detection limit. The variation in these data is large and it should be demonstrated in future larger trials whether any shift in concentration is caused by true differences in human metabolic systems or by measurement errors.

Several (mechanistic) issues remain to be clarified in the future, especially in cattle consuming a large amount of phytosterol -rich feed. There is still debate about the implications of the detection of boldenone in cattle and veal calves' urine. Questions that are raised focus among others on whether boldenone is endogenously synthesized or indeed illegally administered. Also, detection methods might have been improved over time. Another theory that has been proposed is that due to bacteria in feed or a different feed composition (more sterols), the side chain at the c17 position in the D-ring can be broken and the sterols can be formed into anabolic steroids such as

boldenone or testosterone. One should bear in mind that in human food we do not expect this type of bacteria, but the implications for humans in the light of beef consumption needs more research.

To our knowledge, this study is the first to investigate the association between phytosterol (β-sitosterol) consumption and urinary boldenone concentration in humans. And although our pilot study only aimed at hypothesis generation, we very well realize that there are limitations to our pilot study. We had a limited number of participants who only consumed the recommended dose of physterol-enriched margarine, during a limited amount of time. But as the metabolic effects of physiological amounts of phytosterol-enriched margarines are normally observed within one day (Ellegard and others 2005) we are of the opinion that the duration of our pilot had been sufficiently long. Within the scope of the hypothetical aim of this pilot, it was not necessary to collect representative 24h urine samples to take into account variations in urine volume. Therefore, spot urine samples were appropriate to investigate whether boldenone is detectable in urine. Although no levels of boldenone were found, we are of the opinion that in theory it would have been possible to detect sterols and boldenone in human urine samples. Given a mean urine excretion of 1,5 l/day, a phytosterol intake of 2 g/day, and a mean intestinal absorption of 2% (see introduction section), the theoretical maximal concentration of sterols in urine is a factor 300 higher (2% of 2 g sterols = 40 mg/1,5 l = ~30mg/l) than the detection limit of 0.1 µg/l. We did not have any compliance measure of the phytosterol-enriched margarine consumption, but we do not have any indications that among our 10 female volunteers who were contacted regularly during the study period there were any non-compliant subjects.

Our main focus was the detection of urinary boldenone. Despite the limitations described above we conclude that within our setting consumption of the recommended amounts of phytosterol -enriched margarines, does not lead to detectable levels of the anabolic steroid boldenone in women. Further research on boldenone formation is dependent on the observations in cattle and veal calves in relation to beef and veal consumption. Also, the differences in the exact metabolic mechanisms between humans and cattle, and among humans themselves with regard to testosterone are topics of further research.

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Table 1. Overview of the m/z of the different TMS-compounds measured.

Compound	m/z	Retention time (min.)
Cholesterol	368	13.08
Campesterol	382	13.43
Sitosterol	396	13.68
Stigmasterol	394	13.50
Coprostanol	370	12.66
Cholesterol-D6	374	13.08

Table 2. Overview of the m/z of the different (HFB) compounds measured.

Compound	m/z	Retention time (min.)
ß-Boldenone	678	12.97
Boldenone-D3	681	12.95
α-Boldenone	678	13.30
α-testosterone	680	12.81
ß-testosterone	680	13.29
androsterone	486	15.02
etiocholanolone	486	15.11
α-androsteron	486	15.83
testosterone-D2	682	13.28

Table 3. Urine levels of boldenone and phytosterols in 10 healthy human volunteers and a sitosterolemia patient

	Volunteers			Patient with sitosterolemia[1]
	Baseline	**Day 3-4**	**Day 7**	
β-boldenone (µg/l)	nd	nd	nd	nd[2]
α-boldenone (µg/l)	nd	nd	nd	nd
Campesterol (µg/l)				
mean ± sd	0.8 ± 0.3	0.9 ± 0.4	0.9 ± 0.3	7.1
median (min-max)	0.7 (0.5-1.5)	0.8 (0.4-1.5)	1.0 (0.5-1.2)	
Stigmasterol (µg/l)				
mean ± sd	0.2 ± 0.2	0.1 ± 0.2	0.3 ± 0.3	1.5
median[3] (min-max)	- (0.0-0.5)	- (0.0-0.3)	- (0.0-0.5)	
β-sitosterol (µg/l)				
mean ± sd	1.1 ± 0.6	0.9 ± 0.5	1.2 ± 0.4	13.5
median (min-max)	1.1 (0.5-2.4)	0.8 (0.5-1.9)	1.1 (0.6-1.9)	

[1] In this patient levels of boldenone and phytosterols were measured once

[2] nd: not detected below limit of detection: i.e. for campesterol, stigmasterol and β-sitosterol and boldenone < 0.1 µg/l

[3] not calculated due to statistical limitations

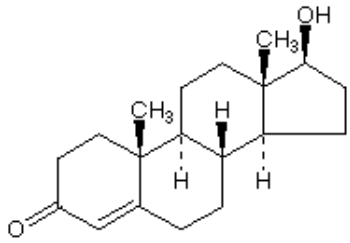
Table 4. Urine levels of testosterone in 10 healthy human volunteers and a sitosterolemia patient

	Volunteers			Patient with sitosterolemia[1]
	Baseline	**Day 3-4**	**Day 7**	
α-testosterone (µg/l)				
mean ± sd	1.5 ± 1.3	3.5 ± 4.1	3.3 ± 1.8	2.8
median (min-max)	0.9 (0.5-3.6)	3.2 (0.0-13.3)	3.5 (0.8-5.2)	
β- testosterone (µg/l)				
mean ± sd	1.3 ± 0.4	3.2 ± 2.1	1.8 ± 0.7	0.1
median (min-max)	1.2 (0.8-1.9)	2.8 (0.8-7.0)	1.9 (1.0-2.7)	
Ratio β- testosterone/ α-testosterone				-
mean ± sd	1.2 ± 0.6	1.4 ± 1.0[2]	0.9 ± 0.8	
median (min-max)	1.0 (0.5-2.1)	1.2 (0.3-2.7)	0.5 (0.2-2.3)	

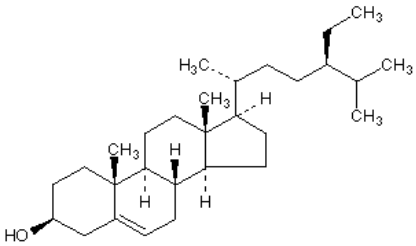
[1] In this patient levels of testosterone were measured once

[2] Mean ratio is 23.2 if one person with values near detection limit is included.

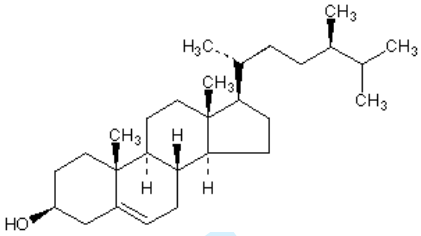
a. Testosterone



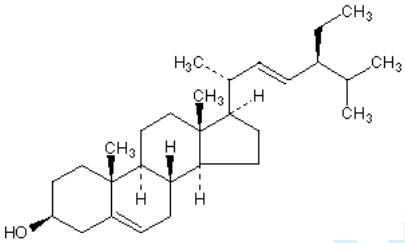
b. ß-sitosterol



c. Campesterol



d. Stigmasterol



e. Boldenone

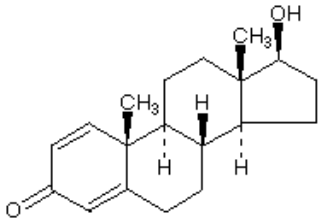


Figure 1. Structures of a.testosterone, b. ß-sitosterol, c. campesterol, d. stigmasterol and e. boldenone