Short Communication

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Sulbutiamine in sports

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Sulbutiamine (isobutyryl thiamine disulfide) is a lipophilic derivative of thiamine used for the treatment of asthenia and other related pathological conditions. It is available over-the-counter in several countries either as a component of nutritional supplements or as a pharmaceutical preparation. The presence of sulbutiamine in urinary doping control samples was monitored to evaluate the relevance of its use in sports. As one of the sulbutiamine metabolites has very close retention time and the same characteristic ion (*m/z* 194) as the main boldenone metabolite, the raw data files generated from the screening for anabolic steroids were automatically reprocessed to identify the samples containing sulbutiamine. It was found that of *ca.* 16 000 samples analyzed in the Russian laboratory during 2009, about 100 samples contained sulbutiamine. It is important to note that most of these samples were collected in-competition, and sulbutiamine concentration was estimated to be greater than 500 ng/ml. This may indicate that sulbutiamine was intentionally administered for its ergogenic and mild stimulating properties.

Keywords: sulbutiamine; doping control; stimulants; sports

Introduction

Sulbutiamine is a vitamin B_1 derivative that readily crosses the blood-brain barrier due to its lipophilic nature and demonstrates glutamatergic as well as dopaminergic properties.[1–3] Clinical indications to use sulbutiamine include chronic fatigue and asthenia,[4–6] though its efficacy is still uncertain. Other published data suggest that sulbutiamine is efficient for the treatment of memory defects[7,8] and erectile dysfunction.[9] Presently, sulbutiamine is available over-the-counter either as a pharmaceutical product (trade names: Enerion and Arcalion – 200 mg sugar-coated tablets both manufactured by Laboratoires Servier, France) or as a component of various nutritional supplements, where it is referred to as 'thiamine di(2-methylpropionate) disulfide' or 'isobutyryl thiamine disulfide'.[10] These supplements are mainly categorized as fat-loss preparations or energy boosters. Therefore, sulbutiamine is likely to be used in sports, either during the restoration period or in-competition.

The aim of present study was to evaluate the pattern and frequency of use of sulbutiamine in different sports by retrospective analysis of previously collected data from the screening of anabolic steroids.

Materials and Methods

Chemicals

Sulbutiamine was isolated from pharmaceutical preparation Enerion (manufactured by Neuilly sur Seine, Paris, France Laboratoires Servier, France). Diethyl ether was obtained from Medkhimprom (Moscow, Russia). β-Glucuronidase from *E. Coli* K12 (solution in 50% glycerol) was purchased from Roche Diagnostics (Mannheim, Germany). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Duren, Germany). A boldenone metabolite, 17β-hydroxy-5β-androst-1-ene-3-one, was obtained from LGC Standards (Wesel, Germany). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Isolation of sulbutiamine from pharmaceutical preparation

One pill of Enerion declared to contain 200 mg of sulbutiamine was carefully stripped to remove the sugar coating and then grinded in a mortar. The powder was extracted with 20 ml of methanol in an ultrasonic bath for 10 min. Following centrifugation at 3200 rpm for 4 min, the supernatant solution was taken to get the solution containing approximately 10 mg/ml of sulbutiamine.

Preparation of urine samples

Urine samples were analyzed according to the standard procedure for conjugated anabolic steroids used in the laboratory. Briefly, to 3 ml of urine was added 1 ml of phosphate buffer (0.8M, pH 6.3) containing 3% of β-glucuronidase and 1.5 µg of methyltestosterone internal standard. Following brief vortexing the samples were placed in an incubator where enzymolysis was allowed to proceed at 55 °C for 60 min. After that 1 ml of carbonate buffer (15% solution of $K_2CO_3/KHCO_3$ 1:1 w/w, pH 10) was added and the samples were extracted with 5 ml of diethyl ether by rigorous vortexing in the presence of Na_2SO_4 as a salting out agent. After centrifugation at 3200 rpm for 4 min the aqueous layer was frozen in a low-temperature bath (−30 °C) and the ethereal extract was poured out into another test tube followed by evaporation at 40 °C under nitrogen flow. The dry residue was treated with freshly prepared MSTFA/NH_4I/dithiotreitol mixture (1000/2/1.5 v/w/w) at 70 °C for 30 min. When the urinary free fraction was analyzed, adding β-glucuronidase and incubation at 55 °C were omitted with all other steps being the same.

GC-MS analysis

The gas chromatographic analyses were carried out on the system comprising a 6890N gas chromatograph coupled to a 5973inert

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mass spectrometer (Agilent, Palo Alto, CA, USA). The separation was achieved on a J&W HP-Ultra 1 column (17 m × 0.20 mm × 0.11 µm) applying temperature programming as follows: 180 °C (0 min), heating at 4 °C/min to 236 °C (0 min), and heating at 20 °C/min to 310 °C (4.5 min). Two µl injections were done using autoinjector 7683 (Agilent, Palo Alto, USA) at 300 °C in the split mode (1 : 20) with a carrier gas flow rate set to 0.6 ml/min (helium 99.999%). Transfer line temperature was 300 °C, the ion source was held at 230 °C, quadrupole temperature 150 °C. The mass spectrometer was operated in the fullscan and SIM modes.

Results and Discussion

Since 2007 we have started to notice that a large peak was occasionally present in the window of the main boldenone metabolite, 17β-hydroxy-5β-androst-1-ene-3-one, with the same *m/z* ratio of 194 (detected as the trimethylsilyl derivative by a single quadrupole GC-MS; Figure 1A). It took several months to discover that this peak corresponded to one of the metabolites of sulbutiamine, a vitamin-like compound with a non-steroidal structure (Figure 2A). The identification was made incidentally when one of the employees in our laboratory got a medical prescription for sulbutiamine to treat asthenia. After providing the informed consent, the person donated urine for analysis. In this urine a large peak with the same spectrum was detected, as observed many times before in routine samples. Our experiments have demonstrated that the detection times were typically 2 to 5 days depending on the intake regime (data not shown).

Taking into account that the recommended daily dose of sulbutiamine is up to 600 mg, concentration of its metabolites in urine is frequently abundant. We have found two peaks with the same base ion at *m/z* 194 present in the sulbutiamine post-administration urine, which were tentatively identified as *mono*-desisobutyryl (M1) and *bis*-desisobutyryl (M2) metabolites of sulbutiamine (Figures 2C and 2B, respectively). The second metabolite eluting in between of the TMS-enol derivatives of principal urinary steroids, androsterone and etiocholanolone, was found less abundant (Figure 3). As the mass spectra of the metabolites are almost identical, it is difficult to unambiguously attribute the correct structure to each metabolite. At this point it is worth mentioning that both metabolites are mainly glucuroconjugated (M2 to the lesser extent), whereas the sulbutiamine parent compound was not detected in urine by

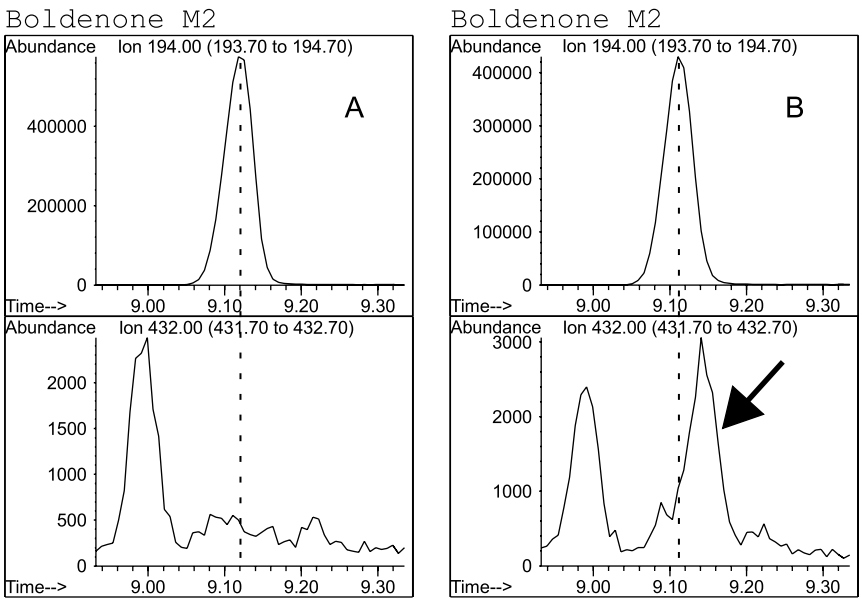

Figure 1. Fragment of a printout from the screening for anabolic steroids: (**A**) abundant signal in the boldenone metabolite window, (**B**) the same urine spiked at 10 ng/ml with the main boldenone metabolite.

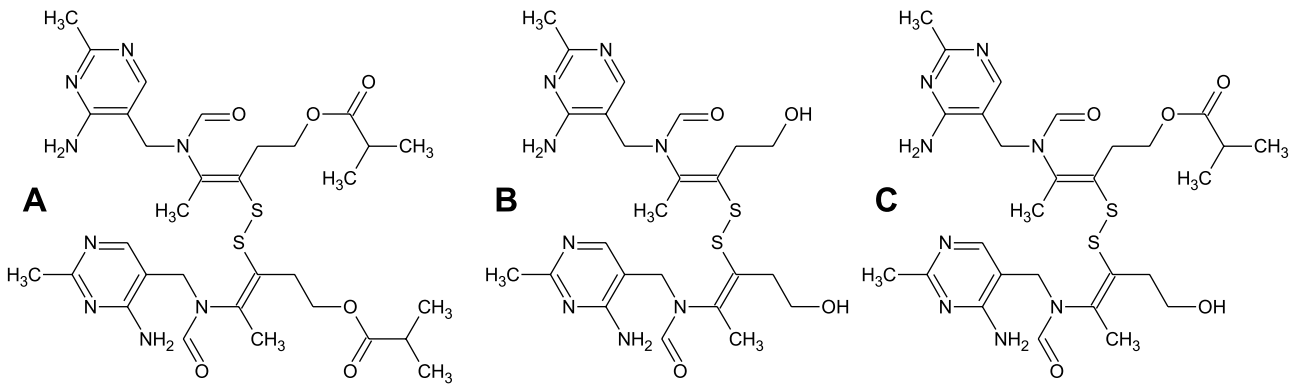

Figure 2. Sulbutiamine (**A**) and its tentative metabolites, *bis*-desisobutyryl sulbutiamine (**B**) and *mono*-desisobutyryl sulbutiamine (**C**).

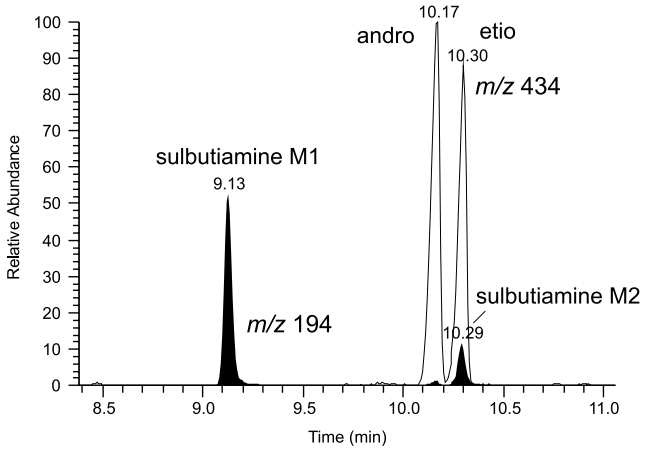

Figure 3. RIC showing sulbutiamine metabolites and Andro/Etio on the same scale (as TMS/TMS-enol derivatives).

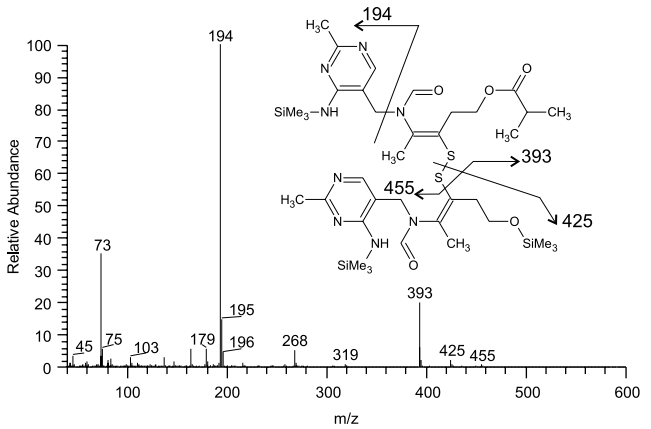

Figure 4. Mass spectrum of TMS ether of sulbutiamine metabolite M1 and proposed fragmentation pathways.

GC-MS. The mass spectrum of metabolite M1 and proposed fragmentation pathways are shown in Figure 4. The structure of both metabolites was hypothesized based on the fragmentation pattern of TMS and *tert*-butyldimethylsilyl derivatives (data not shown).

It is worth noting that the accurate mass of a fragment of boldenone metabolite TMS derivative is 194.1127 ($C_{11}H_{18}OSi$), whilst that of sulbutiamine metabolite is 194.1113 ($C_9H_{16}N_3Si$). This difference of 1.4 ppm is indistinguishable by conventional magnetic sector high resolution mass spectrometers (HRMS), which cannot provide the resolution high enough to separate these two ion species.

Using a simple macro written in the Hewlett-Packard Chem-Station language, we reprocessed the raw datafiles from the screening for anabolic steroids collected during 2009 to find out how often and in what disciplines sulbutiamine was used. As an outcome the macro produced the list of datafiles (i.e. samples) where abundance of the ion at *m/z* 194 (peak height) was higher than the predefined value of 60 000 arbitrary units. This threshold was arbitrarily selected as a compromise between too many false positives and false negatives. Afterwards, the selected analytical reports retained in the laboratory were manually checked for the consistency of this automatic algorithm. The second criterion was the presence of the ions at *m/z* 216 and 268 in the window of epimetendiol. Therefore, this was checked for every sample

Table 1. Summary data on sulbutiamine findings during 2009. IC: in-competition; OOC: out of competition. Note: only sports with findings are listed

	Samples analyzed		Sulbutiamine findings	
Sport	IC	OOC	IC	OOC
Athletics	1217	1492	23 (1.9%)	3 (0.2%)
Biathlon	348	303	2 (0.6%)	0
Boxing	189	214	3 (1.6%)	0
Cross country skiing	309	203	2 (0.6%)	1 (0.5%)
Cycling	764	197	10 (1.3%)	0
Handball	0	227	0	1 (0.4%)
Gymnastics	137	202	2 (1.5%)	2 (1%)
Canoe/kayak	422	248	3 (0.7%)	2 (0.8%)
Judo	234	337	6 (2.6%)	0
Speed skating	199	255	8 (4%)	0
Swimming	354	423	13 (3.7%)	1 (0.2%)
Finswimming	23	0	2	0
Luge	112	123	1 (0.9%)	0
Rriathlon	51	11	5	0
Football	248	420	2 (0.8%)	0
Ice hockey	172	408	4 (2.3%)	0
Short track	52	82	0	1
Bobsleigh	0	61	0	1
Rowing	320	285	2 (0.6%)	0
Total	**5151**	**5491**	**88 (1.7%)**	**12 (0.2%)**

before counting it for the statistics. If not disposed of, the urine samples with sulbutiamine findings were then re-analyzed given the athlete agreed to use the remainder of his/her sample for the anti-doping research.

It should be clarified that another pharmaceutical, trimetazidine, also manifests itself in the same boldenone metabolite window, but this is usually accompanied by a peak in the window of methyltestosterone metabolites (17α-methylandrostanediols) with *m/z* 143 and 255. When such a sample was detected by the macro and there was no opportunity to re-analyze the sample, it was not taken into account.

In total *ca.* 16 000 data files were checked for the presence of sulbutiamine metabolite. As is shown in Table 1, most of sulbutiamine findings were observed for the samples collected in-competition. The percentage per sport given in brackets was calculated when at least 100 samples were available. Though our data may include multiple samples for the same athlete collected throughout the year, or different athletes supervised by the same coach/doctor, it clearly demonstrates the predominant in-competition pattern of use of this compound.

It is worth noting that sulbutiamine, being a part of the athlete's pharmacological support, could hamper the detection of boldenone metabolite due to the coelution when a single quadrupole GC-MS instrument is used for the screening analysis. As the example, Figure 1B shows the print-out where boldenone metabolite was spiked to the sulbutiamine excretion urine at the level of 10 ng/ml.

Conclusion

About 90% of all sulbutiamine findings are related to the samples collected in-competition, and this might point out that

sulbutiamine was deliberately administered for its stimulating effect. The disciplines with the most frequent findings include speed skating, cross country skiing, swimming, cycling, triathlon, ice hockey, gymnastics, and athletics, i.e. are mostly represented by endurance sports.

The anti-doping laboratories should also be aware that the presence of sulbutiamine metabolites in urine may potentially complicate the detection of boldenone.

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