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Detection of the arylpropionamide-derived selective androgen receptor modulator (SARM) S-4 (Andarine) in a black-market product

Non-steroidal and tissue-selective anabolic agents such as selective androgen receptor modulators (SARMs) represent a promising class of therapeutics for the treatment of various diseases such as sarcopenia or cancer cachexia. Advanced compounds of SARMs are based on an arylpropionamide-derived structure and leading drug candidates have successfully completed phase-II-clinical trials. Although none of these therapeutics have been approved, their performance-enhancing qualities and the black-market availability of these products makes them a viable target for misuse in the athletic community. In 2008, SARMs were added to the Prohibited List established by the World Anti-Doping Agency (WADA). That SARMs are the subject of misuse even without clinical approval was proved for the first time by the detection of the drug candidate Andarine (also referred to as *S-4*, S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide), advertised, sold and supplied via the Internet. The oily liquids, declared as green tea extracts and face moisturizer, were assayed using state-of-the-art analytical procedures and S-4 was found at concentrations of approximately 150 mg/mL. The authenticity of the product was demonstrated in comparison to reference material by liquid chromatography, high resolution/high accuracy (tandem) mass spectrometry using positive and negative electrospray ionization, and comparison to reference material. Moreover, an impurity resulting from poor product purification was detected, accounting for approximately 10% of S-4. This consisted of 2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-3-(4-nitro-3-trifluoromethyl-phenylamino)-propionamide.

The ease of purchasing non-approved drug candidates that could potentially increase athletic performance demonstrates the need to operate proactively in the continued fight against doping. The early inclusion of emerging drugs into routine sports drug testing procedures is a key element of preventive doping research, limiting the options for cheating athletes who aim to undermine the doping control system.

Keywords: anabolic; doping; mass spectrometry; preventive; SARMs

Introduction

Novel non-steroidal therapeutics with anabolic effects have been developed and studied for more than a decade following the seminal findings of Dalton *et al*. regarding arylpropionamide-derived substances.[1] Numerous drug candidates of various chemical classes were reported to possess selective androgen receptor modulator (SARMs)-like properties,[2,3] such as selectivity for particular tissues and organs (for example, muscle, bone, and prostate)[4–6] as well as a specific activity representing either agonists or antagonists at particular androgen receptors.[7] Most of these substances proved effective in animal models and a few lead drug candidates have demonstrated promising results in advanced clinical trials that aim to treat age-related maladies and to counteract symptoms of severe diseases such as sarcopenia and cancer cachexia.[8] To date, however, none of these therapeutic agents has received clinical approval and none has been launched as a pharmaceutical product. Nevertheless, SARMs have been prohibited in sports since January 2008 by the World Anti-Doping Agency (WADA)[9] due to their potential misuse. Detection methods for intact SARMs as well as selected metabolites have been established using gas chromatography-mass spectrometry (GC-MS)[10] as well as liquid chromatography-tandem mass spectrometry (LC-MS/MS).[11–17]

The proactive and preventive antidoping approach proved correct and necessary with a recent finding of a non-approved arylpropionamide-derived SARM, Andarine[8] (S-4, chemical structure: S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide (Figure 1). This product was found to be freely available on the Internet at a discount price of 100 USD. In order to probe for authenticity and evidence that non-steroidal anabolic agents lacking clinical approval are distributed and potentially misused in sports, a sample of the advertised substance was analysed using state-of-the-art mass spectrometric approaches with high resolution/high accuracy (tandem) mass spectrometry.

Experimental

Reference material and black-market product

The compound S-4 was synthesized as reference material in our laboratory according to literature data[18–20] and was characterized by mass spectrometry and nuclear magnetic resonance spectroscopy as described earlier.[11]

The product supposedly containing S-4 was purchased via the Internet. Two bottles with approximately 15 mL of an oily liquid

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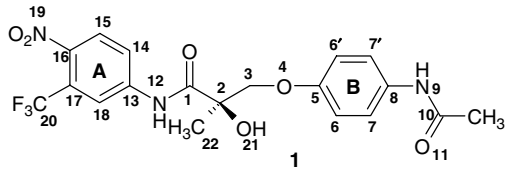

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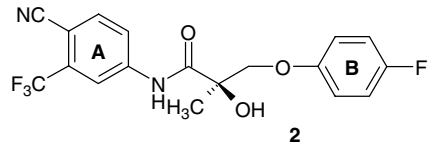

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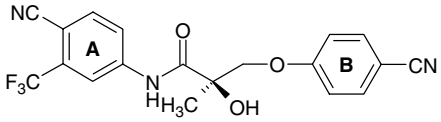

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Figure 1. Chemical structures of arylpropionamide-derived SARMs S-4 (**1**, Andarine), S-1 (**2**), and S-22 (**3**).

were received and analysed for active ingredients and impurities by means of LC-MS/MS with high-resolution/high-accuracy mass spectrometry (see below).

Liquid chromatography – (tandem) mass spectrometry

Characterization and quantitation of the compound detected in the oily liquid was accomplished by means of high resolution/high accuracy (tandem) mass spectrometry. A Thermo Scientific Accela liquid chromatograph (Bremen, Germany) was equipped with a Hypersil Gold analytical column (50 × 2.1 mm, 1.9 µm particle size) and interfaced to a Thermo Scientific Exactive mass spectrometer using electrospray ionization (ESI) in positive and negative modes. LC solvents were 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The flow rate was set to 250 µL/min. Gradient elution was conducted starting at 90% A, decreasing to 0% A in 8 min, maintaining 0% A for further 2 min followed by re-equilibration at 90% A. The ionization voltage was +3.5 kV or −3.0 kV; the capillary temperature was set to 290 °C, and three MS settings were used throughout the analytical runs: (1) full scan MS from *m/z* 50–2000 at a resolution of 25 000 (FWHM), (2) full scan MS (*m/z* 50–2000, resolution set to 10 000) with higher energy collision-induced dissociation (HCD) set to 20 V, and (3) full scan MS (*m/z* 50–2000, resolution set to 10 000) with HCD set to 50 V. Gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany). Elemental compositions of protonated and deprotonated analytes were determined with mass accuracies <5 ppm ensured by external calibration.

Product ion mass spectra were recorded in positive and negative ionization mode using a Thermo Scientific LTQ-Orbitrap mass spectrometer. Accurate masses derived from MS^n experiments

Table 1. Elemental compositions of diagnostic product ions obtained from positive ESI and CID using high resolution/high accuracy MS^n experiments

Precursor ion (*m/z*)									
MS^2	MS^3	MS^4	Elemental comp. (exp.)	Error (ppm)	CE[a]	Product ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
442.1220			$C_{19}H_{19}O_6N_3F_3$	−0.1	14	422.1157	$C_{19}H_{18}O_6N_3F_2$	−0.2	HF
						400.1116	$C_{17}H_{17}O_5N_3F_3$	0.2	C_2H_2O
						396.1293	$C_{19}H_{19}O_4N_2F_3$	−0.4	NO_2
						354.1084	$C_{18}H_{16}O_5N_3$	0.0	H_2O, HCF_3
						206.0810	$C_{11}H_{12}O_3N$	−1.3	HF, $C_8H_6O_3N_2F_2$
	422.1154		$C_{19}H_{18}O_6N_3F_2$	−0.9	25	402.1093	$C_{19}H_{17}O_6N_3F$	−0.6	HF
						382.1030	$C_{19}H_{16}O_6N_3$	−0.8	2 × HF
						354.1082	$C_{18}H_{16}O_5N_3$	−0.7	H_2O, CF_2
						206.0809	$C_{11}H_{12}O_3N$	−1.3	$C_8H_6O_3N_2F_2$
		354.1082	$C_{18}H_{16}O_5N_3$	−0.7		312.0979	$C_{16}H_{14}O_4N_3$	−0.1	C_2H_2O
						295.0713	$C_{16}H_{11}O_4N_2$	−0.3	C_2H_2O, NH_3
						165.0656	$C_8H_9O_2N_2$	−1.3	$C_{10}H_7O_3N$
						123.0551	$C_6H_7ON_2$	−1.7	C_2H_2O, $C_{10}H_7O_3N$
	400.1115		$C_{17}H_{17}O_5N_3F_3$	0.0	15	360.0989	$C_{17}H_{15}O_5N_3F$	−0.1	2 × HF
						354.1186	$C_{17}H_{17}O_3N_2F_3$	0.1	NO_2
						166.0860	$C_9H_{12}O_2N$	−1.3	$C_8H_5O_3N_2F_3$
		354.1188	$C_{17}H_{17}O_3N_2F_3$	0.6	15	246.0737	$C_{11}H_{11}O_2NF_3$	0.2	C_6H_6ON
						109.0521	C_6H_7ON	−1.5	$C_{11}H_{10}O_2NF_3$
	396.1293		$C_{19}H_{19}O_4N_2F_3$	−0.4	13	378.1188	$C_{19}H_{17}O_3N_2F_3$	0.5	H_2O
						246.0737	$C_{11}H_{11}O_2NF_3$	0.2	$C_8H_8O_2N$
						151.0627	$C_8H_9O_2N$	−0.8	$C_{11}H_{10}O_2NF_3$
	354.1084		$C_{18}H_{16}O_5N_3$	0.0	17	312.0979	$C_{16}H_{14}O_4N_3$	−0.1	C_2H_2O
						295.0713	$C_{16}H_{11}O_4N_2$	−0.3	C_2H_2O, NH_3
						165.0656	$C_8H_9O_2N_2$	−1.3	$C_{10}H_7O_3N$
						123.0550	$C_6H_7ON_2$	−2.0	C_2H_2O, $C_{10}H_7O_3N$

[a] CE = collision energy (arbitrary units).

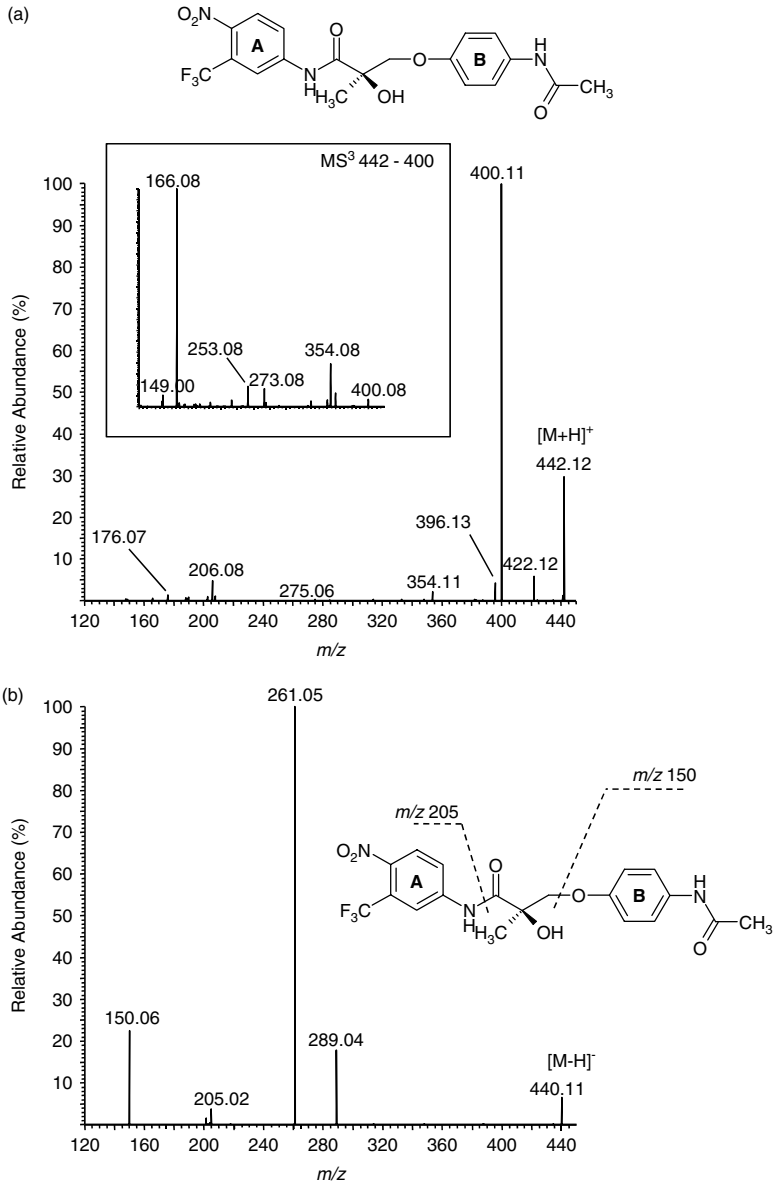


Figure 2. Electrospray ionization product ion mass spectra of the protonated (a) and deprotonated (b) molecules of S-4 as determined in the black-market product.

were used to substantiate the structure of the observed analyte (S-4) and the synthesis byproduct.

Semi-quantitative LC-MS measurements were achieved by preparing a calibration curve of S-4 reference compound in acetonitrile (10–90 μg/mL, 5-point calibration) and analysing a 1/3000 dilution of the oily liquid in acetonitrile. Aliquots of both bottles were assayed in triplicate.

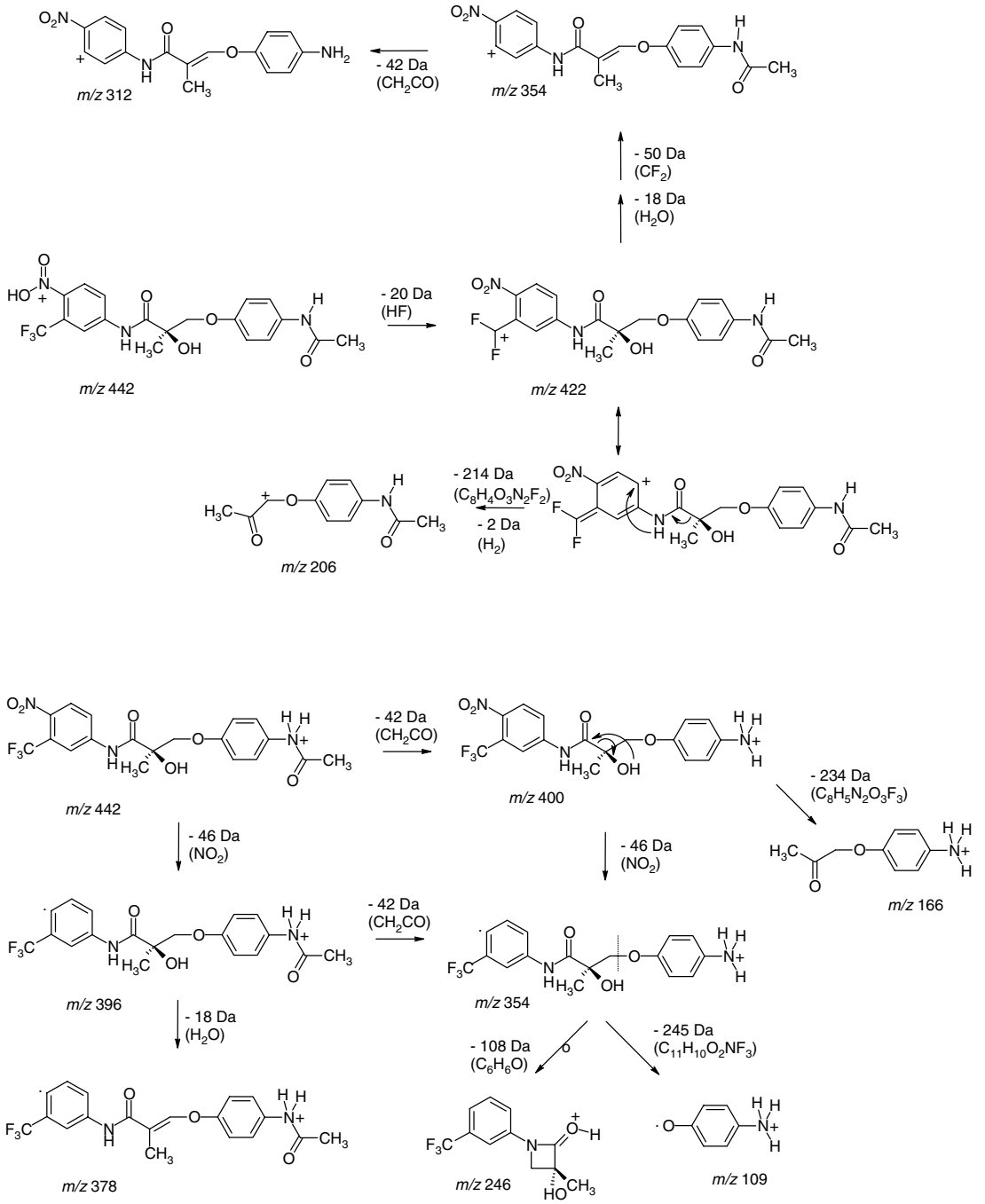
Results and Discussion

S-4 represents one of several structurally related arylpropionamide-derived SARMs (Figure 1), some of which have undergone extensive pharmacokinetic and metabolism studies using *in vitro* and animal models.[21–25] Few of these candidates have successfully completed advanced clinical trials. They demonstrate great promise for the treatment of various debilitating diseases but none has yet received clinical approval.

Even without an official launch, S-4 is available on an Internet website as a bottled solution with the declaration that the product is *not* intended for human use. Due to the potential misuse of such compounds when being available unrestrictedly and the well documented fact that numerous counterfeit products are sold on the so-called black market, one unit (30 mL) was purchased online and delivered in a box labelled as containing face moisturizer and green tea extract. The sealed bottles did not declare any content and no further documents accompanied the package.

LC-MS(/MS) analysis

LC-MS(/MS) analysis of the oily solution purchased online revealed the presence of S-4 at approximately 150 mg/mL of the oily product with equal amounts in each container, yielding a total



Scheme 1. Suggested dissociation pathways of S-4 after protonation and collisional activation.

of 4.5 g of the SARM. The active ingredient was identified and characterized by (1) its elemental composition (as determined by high resolution/high accuracy mass spectrometry – Table 1), (2) comparison to synthesized reference material regarding retention time and product ion mass spectrum, and (3) elucidation of its mass spectrometric behaviour. In Figure 2, the product ion mass spectra of the protonated and deprotonated compound S-4 as observed in the purchased product are depicted proving the authenticity of the substance.

The dissociation pathways of S-4 after negative electrospray ionization have been described in depth in an earlier report[17] and match the pathways found in our present study. Further, fragmentation routes after positive ESI were investigated by means of MS^n experiments and the accurate masses of the observed product ions were determined (Table 1). These analyses revealed complex dissociation behaviour upon collisional activation of the protonated molecule as summarized in Scheme 1. Most prominent product ions were observed at *m/z* 422, 400, 396, 354, and 206 (Table 1), with the first mentioned being obtained from the elimination of hydrogen fluoride (HF, −20 Da). Further dissociation of *m/z* 422 yielded abundant signals at *m/z* 354 and 206 due to the losses of water (−18 Da) plus CF_2 (−50 Da) and the substituted anilide moiety plus molecular hydrogen (−216 Da, $C_8H_5NF_2$), respectively, yielding the proposed structures illustrated in Scheme 1. The ion

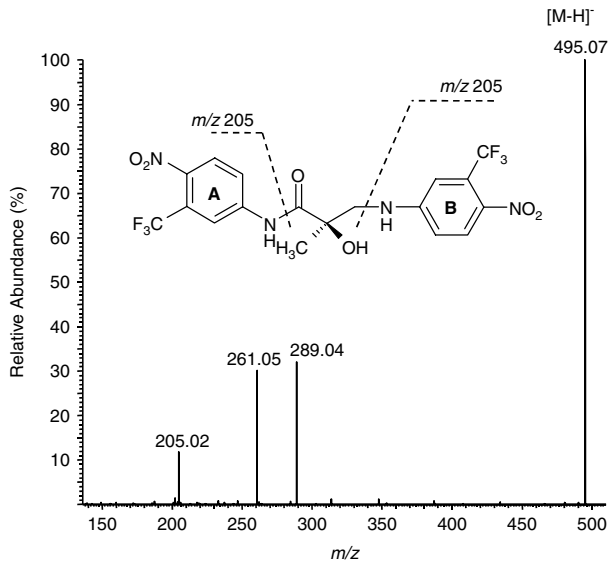


Figure 3. Electrospray ionization product ion mass spectrum of the deprotonated molecule of a drug impurity presumably resulting from marginal isolation of synthesis intermediates.

at *m/z* 354 (having an elemental composition of $C_{18}H_{16}N_3O_5$) was shown to contain an intact acetyl function by means of MS^3 experiments, which resulted in the loss of ketene yielding *m/z* 312 (Scheme 1). Alternatively, the precursor ion at *m/z* 442 eliminated ketene (−42 Da) and nitrogen dioxide (−46 Da) to produce the ions at *m/z* 400 and 396, respectively. Due to consecutive losses of these species, their common product ion was found at *m/z* 354, which comprised a different elemental composition ($C_{17}H_{17}N_2O_3F_3$) from the ion resulting from *m/z* 422 ($C_{18}H_{16}N_3O_5$, Table 1). In MS^n experiments, the homolytic cleavage of the C-O bond (carbon 3 and oxygen 4) of *m/z* 354 was observed yielding the product ions at *m/z* 246 and 109, with the first mentioned necessitating a hydrogen migration and, presumably, a formation of an azetidin-2-one ring structure (Scheme 1). Another abundant ion formed from *m/z* 400 was observed at *m/z* 166, the origin of which is proposed to be the loss of 4-nitro-5-trifluoromethylphenyl-formamide (−234 Da).

Besides the detection of the active ingredient S-4, a byproduct with a molecular weight of 496 Da was observed (Figure 3) at an estimated concentration of 15 mg/mL. Its elemental composition as deprotonated species was determined as $C_{18}H_{13}N_4O_6F_6$ (error: 1.8 ppm), and its product ion spectrum outlined a dissociation behaviour with close analogy to that of S-4; however, the ion at *m/z* 150 (resembling the 4-acetylamino-phenoxy residue) was not generated. The increment of 55 Da compared to S-4 was thus assigned to the B-ring of the molecule and resulted in a product ion at *m/z* 205.0228 (error: 4.1 ppm), which overlapped with the A-ring fragment with identical elemental composition. Considering the route of synthesis, insufficient purification of an intermediate product obviously led to the formation of 2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-3-(4-nitro-3-trifluoromethyl-phenylamino)-propionamide (Figure 3), which was not separated and removed from the intended drug candidate.

Conclusion

The availability of a non-steroidal anabolic agent of the SARM class was demonstrated and the authenticity of the product was proven by state-of-the-art mass spectrometric techniques. Major concerns result from these findings, in particular the concern that this product with considerable anabolic properties is readily available without sufficient research on its undesirable effects; this is especially significant where uncontrolled dosing is applied and drug impurities with unknown effects are present in considerable amounts as observed in the studied material. Such impurities can serve, however, as distinctive feature to differentiate a pharmaceutical product from black-market substances. With regard to sports drug testing it must be stressed that preventive doping research is essential to limit the options of cheating athletes who aim to undermine doping control systems. The present report demonstrates once more that the misuse of therapeutics in early or advanced clinical trials by athletes cannot be dismissed, especially when anecdotal evidence for the misuse of S-4 is frequently discussed in respective Internet-based chat rooms.

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