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Black market products suspected to contain doping relevant ingredients - annual report for 2017

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Abstract

The black market for performance enhancing substances is a popular source for athletes of mass sport. The goal of an optimized body composition leads unfortunately to the consumption of illegally supplied and produced products. The health risks due to poor quality, lack of medical indication, false administration, or the lack of clinical studies are immense.

The products analyzed at the Cologne Anti-Doping Laboratory in order to monitor developments with regard to novel performance enhancing drugs in 2017, comprised original pharmaceuticals as well as products from underground laboratories, which were confiscated by customs authorities. In total, the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed 69 products by means of liquid chromatography - mass spectrometry (LC-MS), gas chromatography - mass spectrometry (GC-MS), and polyacrylamide gel electrophoresis (PAGE) methods. 91% of the compounds considered relevant for doping controls accounted for anabolic agents; 4% accounted for peptide hormones, growth factors, related substances and mimetics, and 5% for hormone and metabolic modulators.

Remarkable findings were the detection of modified GHRP-6 and modified hGH both comprising an altered N-terminus. The administration of synthetic GHRPs results in stimulation of Growth Hormone (GH) secretion, while hGH is a prescription drug used in case of infantine growth disorders and adult growth hormone deficiency. GHRPs and hGH are listed in the WADA Prohibited List and banned for athletes. However, the effects of an additional glycine at the N-terminus of GHRPs or an additional alanine in case of hGH on the human body still remain to be elucidated. Furthermore, the variation of target molecules, with or without intent, represents new challenges for the development of analytical approaches and underlines the importance to monitor the black market concerning emerging compounds.

Introduction

The goals of mass sport athletes are sociality, fun and fitness by exercise, and last but not least optimizing their body´s composition. The black market for performance enhancing drugs, comprising original pharmaceuticals as well as faked products from underground laboratories, arguably aims at providing materials for amateur athletes [1]. In order to monitor developments with regard to novel performance enhancing drugs, the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 69 predominantly confiscated products qualitatively and quantitatively in 2017.

The black market for performance enhancing drugs, including original pharmaceuticals as well as faked products, is a popular source for amateur athletes [1-3].

 ISBN 978-3-86884-044-5

Monitoring developments with regard to novel performance enhancing drugs, the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 69 products qualitatively and quantitatively in 2017. Doping-relevant findings accounted in 99 cases for 35 different drugs (multi-findings included), from which 44% of the ingredients were not or falsely declared.

Experimental

Depending on the formulation (oily solution, lyophilized, etc.), samples were solved or extracted with water, acetic acid (2% aq.), and/or acetonitrile (50:50 *v/v)* and subsequently diluted to yield an adequate concentration of labeled drug content. For gas chromatography, extracted and afterwards dried samples were reconstituted in ethyl acetate, derivatized with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), or a mixture of MSTFA/ethanethiol and ammonium iodide, respectively [1]. To screen the most common target analytes in black market products, high performance liquid chromatography/mass spectrometry (HPLC-MS) experiments were conducted in single-reaction-monitoring (SRM) mode. Anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors could be determined by high performance liquid chromatography / high resolution mass spectrometry (HPLC-HRMS)-experiments in full-scan mode. Qualification and quantification of analytes were obtained by conducting product-ion scans with substance specific fragmentation pathways. For gas chromatography / mass spectrometry (GC-MS) experiments, analytes were derivatized and measured in full-scan mode. Qualitative and quantitative analysis were accomplished by using reference substances and/or reference databases. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics. For the analysis of proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. By bottom-up proteomic approaches including tryptic digestion and nano liquid chromatography/tandem high resolution mass spectrometry, proteinogenic ingredients were identified. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors [1].

Results and Discussion

In 2017, a total of 69 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. As shown in Fig. 2, 91% of the identified doping relevant compounds accounted for anabolic agents (predominantly testosterone esters); 4% accounted for peptide hormones, growth factors, related substances and mimetics and 5% for hormone and metabolic modulators. Falsely labeled products with doping relevant ingredients accounted for 44% (Tab. 1). The analytes, which were currently not doping relevant, were dermatologic agents, vitamins, and amino acids.

 ISBN 978-3-86884-044-5

Drug	Finding	Labelled	Not labelled	
S1 Anabolic agents				
Androstenedione	1		1	
Bromo-Androstendione	1	1		
Boldenone	1		1	
-Undecylenate	2	2		
Clenbuterol	1	1		
Metandienone	4	2	2	
Metenolone				
-Enantate	1	1		
-Acetate	1	1		
Methyldrostanolone	1	1		
Nandrolone				
-Decanoate	4	1	3	
-Phenylpropionate	1		1	
Ostarine	6	2	4	
Oxandrolone	3	3		
Oxymetholone	1	1		
RAD-140	2	2		
SARMs				
LGD-4033	1	1		
Ostarine (MK-2866)	6	2	4	
Stanozolol	3	3		
Testosterone	2	1	1	
-Cypionate	3	2	1	
-Decanoate	5	3	2	
-Enantate	10	5	5	
-Isocaproate	3	3		
-Phenylpropionate	6	3	3	
-Propionate	10	5	5	
Trenbolone				
-Acetate	7	1	6	
-Enantate	4	2	2	
S2 Peptide hormones , growth factors, related substances and mimetics				
modified GHRP-6	1		1	
hGH (Somatropin)	1	1		
modified hGH	1		1	
Ibutamoren (MK-677)	1	1		
S4 Hormone and metabolic modulators				
Anastrozole	1	1		
Clomifene	1	1		
GW-501516 (Endurobol)	2	1	1	
Insulin	1	1		
35 Doping relevant drugs	**99**	**55**	**44**	Doping relevant
Unspecific / other drugs				
Amino acids	2	2		
Vitamins	1	1		
Yohimbine	1		1	
In total 69 products with 38 analytes	**103**	**58**	**45**	Currently not doping relevant

Table 1: Identified drugs in black market products.

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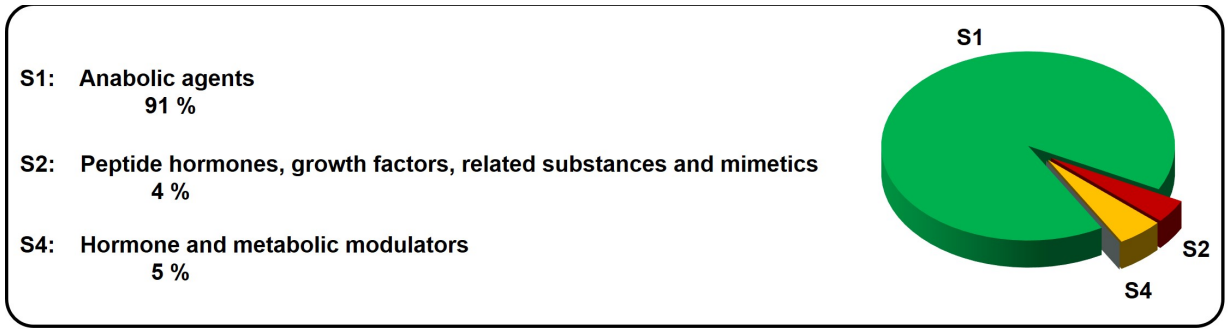

Figure 2: Apportionment of identified doping relevant drugs in analyzed black market products 2017

Remarkable findings were the detection of modified GHRP-6 (Fig. 3) and modified hGH (Fig. 4). The application of synthetic GHRPs was found to stimulate Growth Hormone (GH) secretion [4], while hGH is a prescription drug used in case of infantine growth disorders and adult growth hormone deficiency [5].

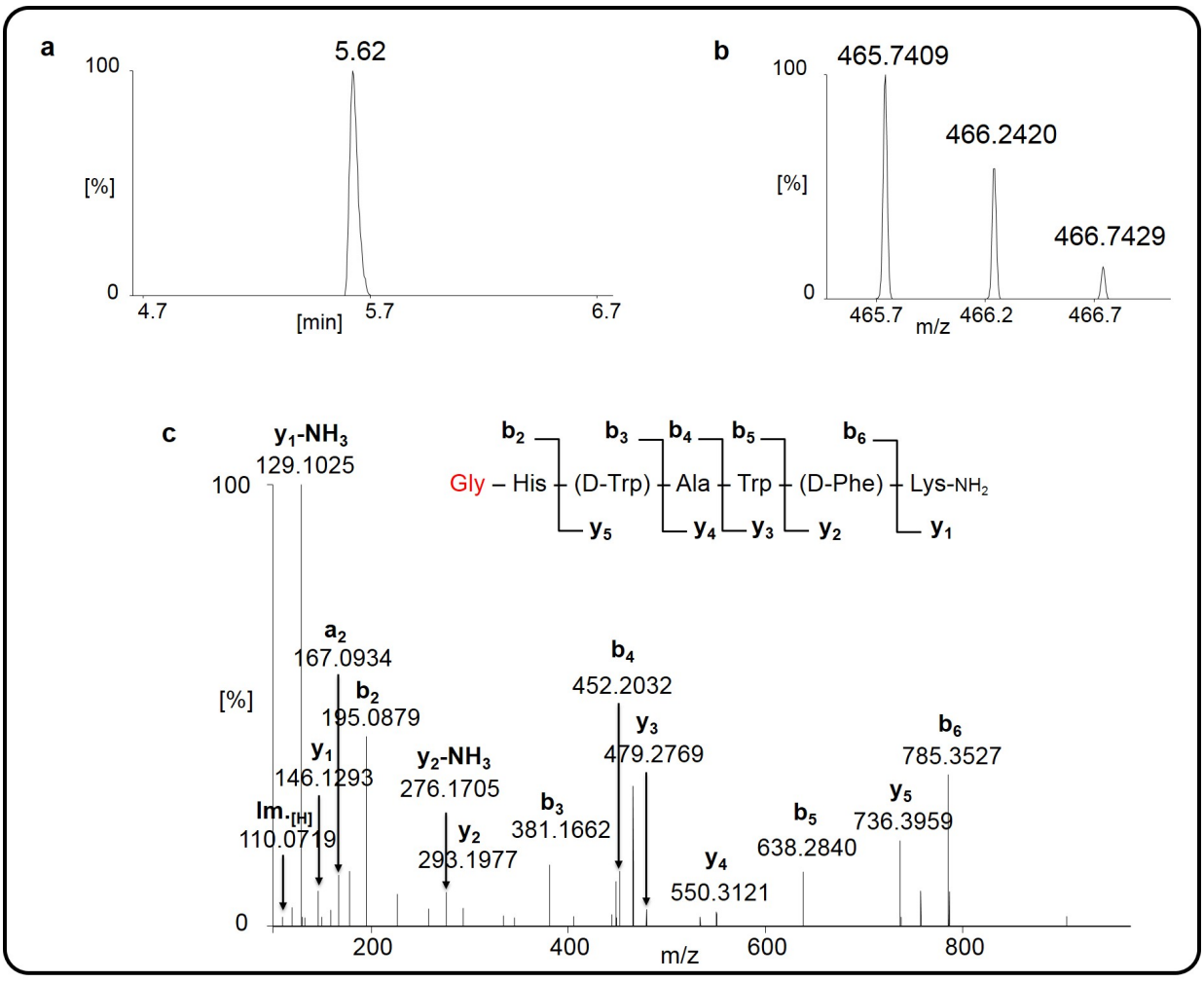

Figure 3: Extracted ion chromatogram of the two-fold charged peptide at m/z 465.7409 $[M+2H]^{2+}$ (a) and corresponding mass spectrum (b). High resolution / high accuracy product ion mass spectrum of modified GHRP-6; generated from $[M+2H]^{2+}$ m/z 465.74. The inset shows the amino acid sequence of modified GHRP-6 with elemental composition $C_{48}H_{59}N_{13}O_7$ and monoisotopic mass of 929.4660 Da; b- and y-ions are labeled, added glycine at N-terminus.(c).

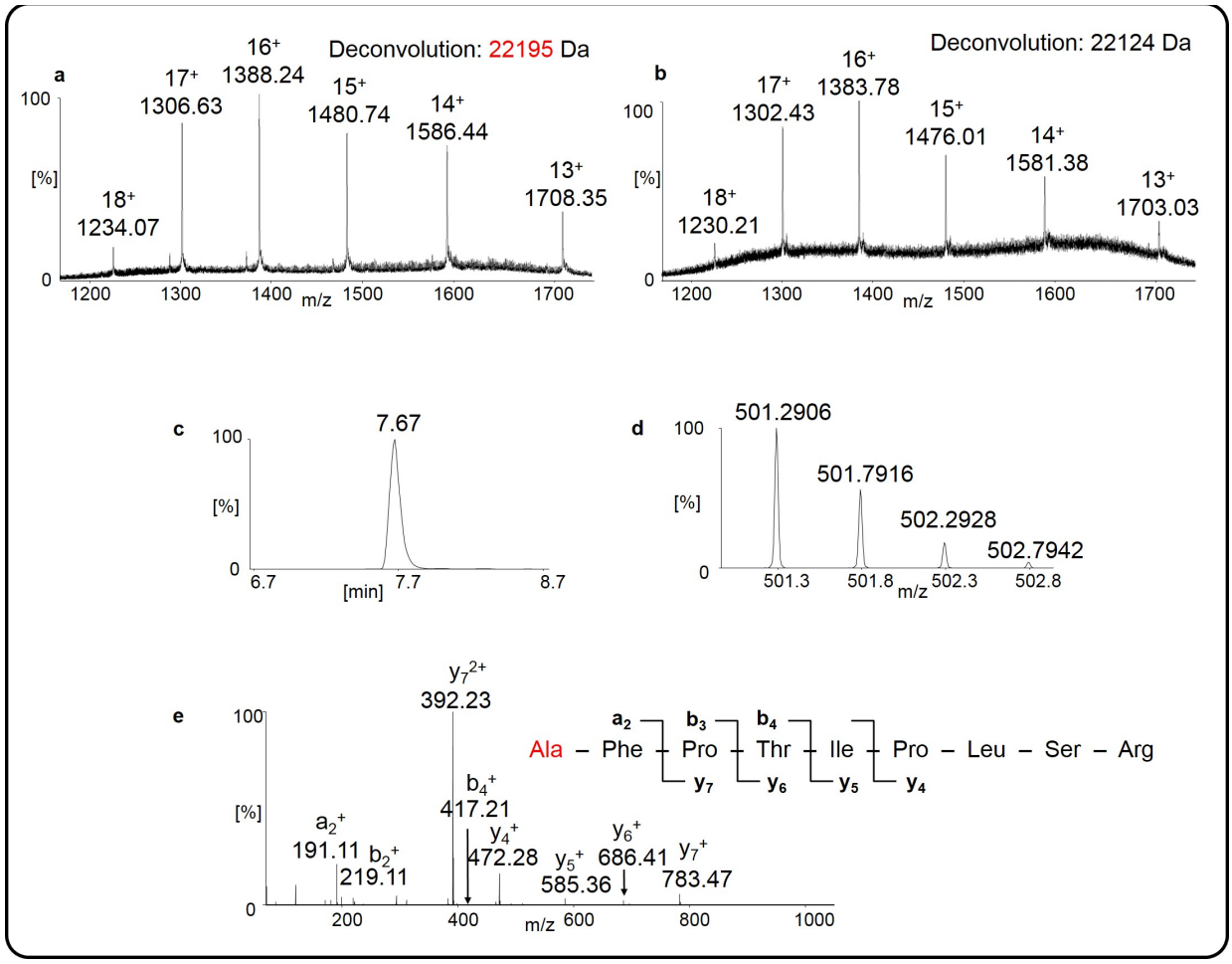

Figure 4: High resolution full MS spectra of the confiscated black market product (a) and the recombinant GH reference (b). Deconvolution yielded approximate molecular masses of 22195 Da for (a) and 22124 Da for (b). Extracted ion chromatogram of the two-fold charged tryptic signal peptide (T1: AFPTIPLSR) at m/z 501.2906 $[M+2H]^{2+}$ (c) and corresponding mass spectrum (d). The diagnostic product ion mass spectrum shows the detection of a-, b- and y-ions from the peptides backbone, it clearly identifies the suggested amino acid sequence (e).

Conclusions

GHRPs and hGH are listed in the WADA Prohibited List and banned for athletes [6]. The shift of physiological effects through modification by addition of an amino acid to the peptide or protein, respectively, is unclear. Furthermore the variation of target molecules, with or without intent, means new challenges for the development of analytical approaches. In the meantime, several GHRP-Glycine-analogues (Gly-GHRP-2, Gly-GHRP-6, and Gly-Ipamorelin) were confiscated, identified and/or charackterized in Germany, Norway, and Poland [5, 7]. This underlines the importance to monitor the black market concerning emerging compounds.

 ISBN 978-3-86884-044-5

References

1. O. Krug, A. Thomas, K. Walpurgis, T. Piper, G. Sigmund, W. Schänzer, T. Laußmann, M. Thevis: Identification of black market products and potential doping agents in Germany 2010-2013 (2014) *Eur J Clin Pharmacol, Vol. 70, 1303-1311*
2. C. Weber, O. Krug, M. Kamber, M. Thevis: Qualitative and Semiquantitative Analysis of Doping Products Seized at the Swiss Border. (2017) *Substance Use & Misuse , Vol 52, (6) 742-753*
3. C. Weber, M. Kamber, V. Lentillon-Kaestner: Are doping substances imported into Switzerland mainly to increase athletic performance? (2016) *Performance Enhancement & Health. Vol 5(2), 66–76.*
4. R.G. Gondo, M.H. Aguiar-Oliveira, C.Y. Hayashida, S.P. Toledo, N. Abelin, M.A. Levine, C.Y. Bowers, A.H. Souza, R.M. Pereira, N.L. Santos, R. Salvatori: Growth hormone-releasing peptide-2 stimulates GH secretion in GH-deficient patients with mutated GH-releasing hormone receptor (2001) J Clin Endocrinol Metab, 86(7):3279-83.
5. O. Krug, A. Thomas, H. Malerød-Fjeld, Y. Dehnes, T. Laussmann, I. Feldmann, A. Sickmann, M. Thevis: Analysis of new growth promoting black market products.
6. https://www.wada-ama.org/sites/default/files/resources/files/2016-09-29_-_wada_prohibited_list_2017_eng_final.pdf
7. M. Poplawska, A. Blazewicz: Identification of a novel growth hormone releasing peptide (a glycine analogue of GHRP-2) in a seized injection vial. *Drug Test Anal*, 2018, 1-6, doi: 10.1002/dta.2467

Acknowledgements

The authors thank, the Federal Ministry of the Interior and the Manfred Donike Institute for financial support.