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Confiscated Black Market Products and Nutritional Supplements with Non-Approved Ingredients Analyzed in the Cologne Doping Control Laboratory 2009

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Doping control laboratories are frequently confronted with new substances that may be misused by athletes. Besides new pharmaceuticals, where method development for their detection is dependant on the availability of the substance and corresponding administration studies, some professional and amateur athletes are using illicit 'black market' products, which either differ from known pharmaceuticals but cause similar effects or still are undergoing clinical trials and are therefore rarely available to doping control laboratories. In the Cologne Doping Control Laboratory, different confiscated products and legally obtained nutritional supplements were analyzed in 2009, and various findings were reported including GH-labelled injection vials without any pharmacologically active content; combinations of products indicating the attempt to mask growth hormone abuse; unpurified long-R^3-IGF-1; nutritional supplements containing the growth hormone releasing peptide-2 (GHRP-2); and ampoules containing the selective androgen receptor modulator Andarine (S-4). This review provides an overview on the substances that were analyzed in 2009. Ingredients relevant for doping control were identified by means of liquid chromatography and mass spectrometry methods. The awareness of new products on the black market and in nutritional supplements is of utmost importance for laboratories to develop detection methods accordingly and screen for new substances as early as possible. **Copyright © 2010 John Wiley & Sons, Ltd.**

Keywords: doping; sport; mass spectrometry; black market; nutritional supplements; S-4; GHRP-2; His-tag

Introduction

The black market provides an alternative source of performance-enhancing substances for cheating athletes. These are usually cheaper than authentic preparations, and substances of a slightly modified structure have been made available as demonstrated; for example, Tetrahydrogestrinone (THG), which was identified during the BALCO scandal in 2003.[1–3] In addition, products which are still in early or advanced clinical trials are frequently offered on the black market, sometimes even years prior to their pharmaceutical launch. These facts bear several dangers. The alteration of molecular structures even to a presumably minor extent may result in considerably different effects and side effects that are unpredictable and unknown to the consumer as well as to the producer. The (mis)use of substances which are not yet approved as pharmaceuticals is as critical; health risks are not foreseeable, especially when the applied amounts and co-administration with other drugs are entirely uncontrolled.

Substances from the black market may be cheap but numerous analyses revealed that several of those did not contain any performance-enhancing ingredients. Because so-called 'underground laboratories' often have neither the knowledge nor aim to manufacture high-quality products that are of a standard similar to that required by pharmaceutical companies nor do they store them adequately,[4] byproducts and analogues with different (if any) effects have been detected in various black market labels. These shortcomings are of utmost relevance if production or purification is more complex, as for example in the case of recombinant peptide hormones.

Nutritional supplements, which can be bought from legal sources and commonly contain substances such as minerals or vitamins, can also contain performance-enhancing substances which are added intentionally or are contaminations from production machines.[5]

The products available on the black market change with the substances available on the pharmaceutical market (or even earlier). In this context, while the market was dominated by anabolic steroids for the last few decades,[6–9] protein- and peptide-based substances seem to make up a bigger part of black market products today, although other alternative compounds such as selective androgen receptor modulators can also be bought illegally.

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Table 1. Products analyzed for prohibited substances from confiscations or nutritional supplements

No.	Label and packing	Source	Identified substances
1a	2 Somatrope (recombinant hGH) injection vials	Obtained from the Anti-Doping Authority of the Netherlands	No substances detected
1b	Eprex 4000, 10000 U/mL, syringe		Recombinant Erythropoietin
1c	Hexarelin, injection vial		Hexarelin
1d	Colourless injection solution in vial, without label		His_6-tagged long-R^3-IGF-I
2a	Hemotropin, tablets	Bought in Cyprus	GHRP-2 (~50 µg/tablet)
2b	Hemogex, drinking solution		GHRP-2 (~9 mg/vial)
3	SARM S4	Bought in the USA on the Internet	Andarine (S-4)
3a	Injection vial without label	Confiscated at Customs	Peptides of long-R^3-IGF-I, mass of the intact molecule different
3b	Injection vial with Chinese label		Recombinant choriongonadotropin (hCG)
4	Injection vial without label	Confiscated by the Customs Criminal Investigation Office	long-R^3-IGF-I
5	Injection vial labelled Geotropin	Confiscated by the German police	Recombinant growth hormone

This short review summarizes the products that were analyzed in the Cologne Doping Control Laboratory in 2009 and gives an overview on the classes of substances and the astonishing small number of products that contain exactly the labelled substance.

Confiscated Products

A number of different products were analyzed from various sources such as customs, police, and national anti-doping authorities or were bought over-the-counter as nutritional supplements. Table 1 gives an overview of the products and the bioactive components that were identified. Most of the commodities contained protein- or peptide-based substances, many of which were not in agreement with their respective labels or contained poorly purified analogues or artefacts. The different products will be presented and four products, namely an injection vial containing unpurified long-R^3-IGF-I as well as two nutritional supplements containing GHRP-2 and a flacon with the selective androgen receptor modulator Andarine (S-4) will be discussed in more detail.

Detection of His-tagged long-R^3-IGF-1 in an injection vial

An unlabelled injection vial was sent to the laboratory together with two injection vials labelled to contain growth hormone, one injection vial of recombinant erythropoietin, and a vial of Hexarelin (Table 1, 1a–1d). The unlabelled vial (Table 1, 1d) contained a transparent injection solution and mass spectrometric analysis yielded the presence of long-R^3-IGF-I as well as a much higher amount (approximately 5–10 times more) of unpurified, His_6-tagged long-R3-IGF-I.[10]

IGF-1 is the main mediator of the anabolic effects of growth hormone and stimulates muscle anabolism and bone growth in childhood. Different variants, such as long-R^3-IGF-I, R^3-IGF-I or Des-(1–3)-IGF-I, were produced to prolong the half-life by changing the binding protein affinities [11–12] but none of these analogues is approved as a therapeutic agent. These different structures provide the doping control laboratories with a xenobiotic target which is different from endogenous IGF-I; detection methods were developed in the past few years.[13]

The identification of the His-tagged analogue was performed by electrophoretic separation, trypsin digestion, and nano-UPLC-high resolution/high accuracy mass spectrometry. The most abundant signal in the sample after dilution in acetic acid and injection into the Orbitrap mass spectrometer after separation of the components resulted from a compound with an intact monoisotopic mass of 10 169.87 Da. Additionally, long-R^3-IGF-I was detected with 9105.38 Da and identified by comparison to a reference standard. The modification of the main component was identified as tryptic peptide with the mass of 1240.563 Da. The MS/MS spectra of the peptide are shown in Figure 1 and clearly identify the peptide as the two C-terminal amino acids of long-R^3-IGF-I, the linker amino acids Leu-Glu and six histidine residues attached to the C-terminus. Affinity tags are usually attached to proteins during synthesis in cells by including the sequence in the expression vector. This enables a very efficient purification of the protein and the tag can be specifically cleaved when attached to the N-terminus. The tagging of a protein at the C-terminus as it was detected in the seized vial is not typical for a recombinant protein that shall be used for administration purposes because there are no specific proteases to cleave His-tags at the C-terminus which means that at least some linker amino acids representing an attached cleavage site for a protease stay at the recombinant protein. C-terminal His-tags are used for biochemical studies when the active site of the protein is at the N-terminus. In such cases the C-terminus is preferred; the protein is purified with the help of the affinity tag and for the following functional, structural or interaction studies the small His-tag was shown to have no significant influence.[14–16] This leads to the assumption that the injection vial ingredient is either a byproduct from biochemical studies and was not produced for injection or that the underground producers were not experienced enough to use the correct vector for their purposes.

Detection of GHRP-2 in different nutritional supplements

Two nutritional supplements containing GHRP-2 were bought in Cyprus. GHRP-2 is a growth hormone releasing peptide (GHRP) that binds to the ghrelin receptor to stimulate endogenous growth hormone release from the pituitary.[17–18] The

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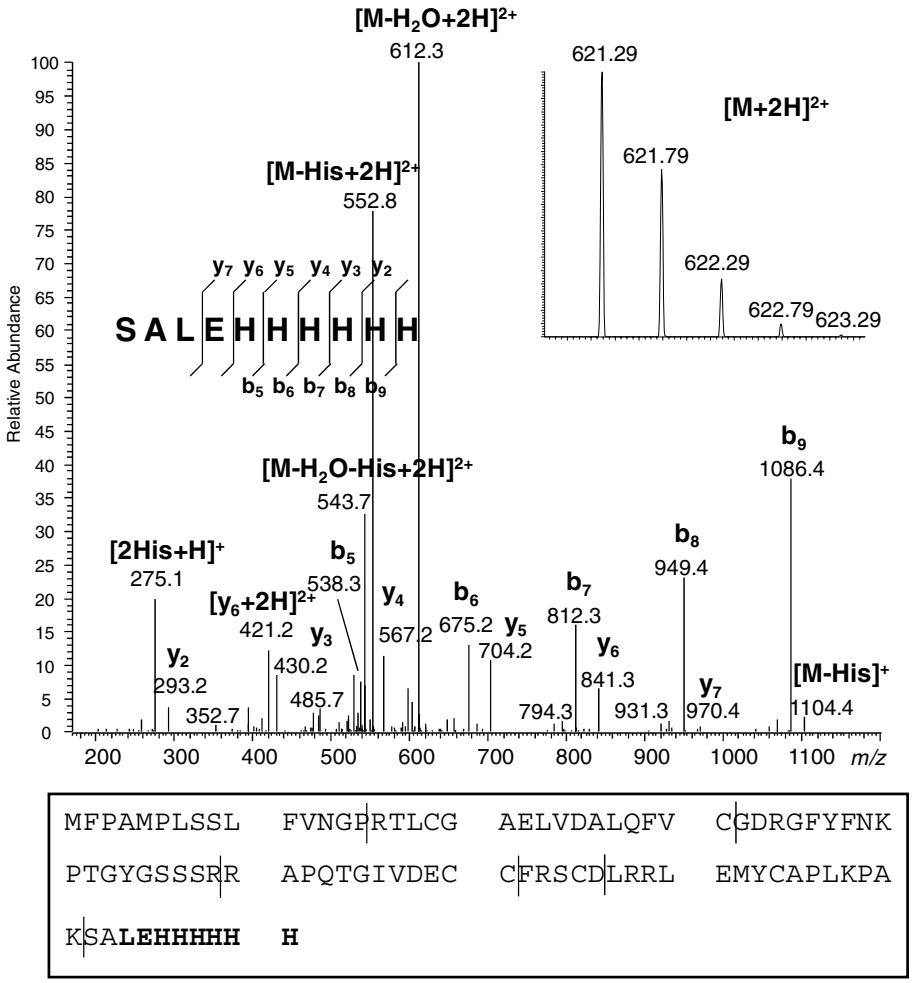

Figure 1. Mass spectrum and MS/MS spectrum identifying the His-tag on the modified long-R^3-IGF-I. The sequence of long-R^3-IGF-1 is presented below the spectrum and the modification is written in bold. Vertical lines indicate trypsin cleavage sites.

performance-enhancing effects are therefore similar to those of growth hormone including muscle growth, stimulation of fat metabolism, or bone growth in adolescents. GHRP-2 is one of many slightly different peptides which are tested for their growth-hormone-releasing properties in clinical studies.[19]

One of the products was labelled to contain GHRP-2 (His-D-β-Nal-Ala-Trp-D-Phe-Lys-OCH_3) and three other natural growth hormone releasers namely Arginine Pyroglutamate, (2S)-2-aminopentanedioic acid and L-alpha glycerylphosphorylcholine (Table 1, 2a). The combination of Arginine and GHRP-2 was described as an especially robust stimulus for growth hormone secretion in administration studies in growth-hormone-deficient children.[20]

In-depth analysis of the tablets for the labelled GHRP-2 resulted in the determination of 50 µg of GHRP-2 per tablet with the correct sequence of D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2 (and not the one printed on the label, *vide supra*). The structure on the label does not belong to GHRP-2 and not to any other growth-hormone-releasing peptide and was not detected in the sample.[21]

The second nutritional supplement that was analyzed for GHRP-2 was labelled to contain active GHRP-2 ester Z-11 (Table 1, 2b). One ampoule of the drinking solution was found to contain approximately 9 mg of GHRP-2 and one ampoule represents one recommended daily dose. Studies with oral application in growth-hormone-deficient children applied around 300–900 µg/kg/day which means that the amount in the nutritional supplement is close to the currently tested pharmaceutical amounts.[17,22] In Figure 2, the MS/MS spectrum of GHPR-2 is shown; the peptide was identified by comparison to a reference substance. Doping with GHRP-2 can currently be detected by screening methods applicable to plasma and urine samples.[23–24]

Detection of Andarine (S-4) for oral administration

Selective androgen receptor modulators (SARMs) are still in clinical studies and Andarine is one of the advanced products with phase II clinical trials completed. They are supposed to replace anabolic androgenic steroids in several disease treatments and, presumably, also in terms of doping practices. SARMs bind to the androgen receptor in a tissue-selective manner, which results in a similar effect to anabolic androgenic steroids but without the commonly observed negative side effects which are produced by metabolites of the steroidal agents generated via the 5-alpha-reductase pathway. This metabolic route is not effective on SARMs being structurally different from steroids.[25] Synthesized products and *in vitro* metabolism studies are the current basis of doping control analytical assays for this group of substances.[26–30]

A glass bottle labelled SARMS S-4 300 mg/mL with the advice that it is not for human consumption was ordered over the

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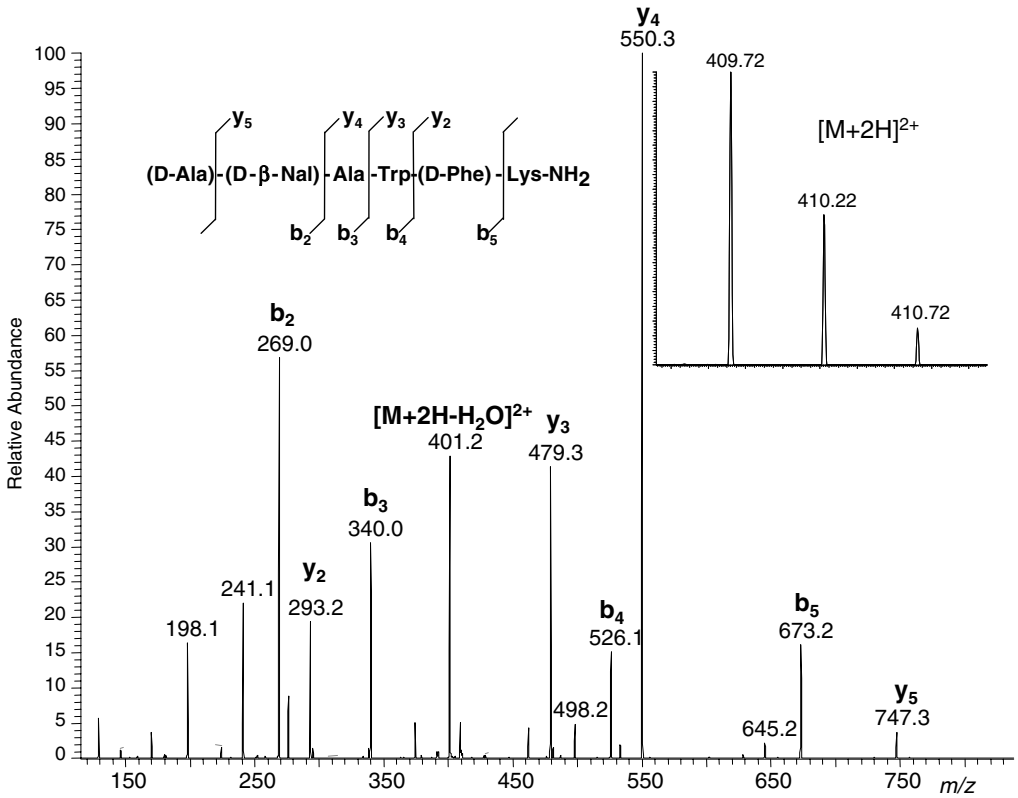

Figure 2. Product ion mass spectrum of GHRP-2 as it was detected in product 2a and 2b. The spectrum as well as the retention time was compared to a reference standard of GHRP-2 for identification.

Internet and arrived as 'Tea Tree Extract' and 'Face Moisturizer' (Table 1, 3). The flacon contained approximately 150 mg/mL of S-4 and additionally a byproduct from the synthesis that was not abolished during purification and accounting for roughly 10% of the amount of S-4.[31] MS/MS spectra of S-4 in positive and negative mode identifying the substance by characteristic product ions are depicted in Figure 3.

A batch of various substances obtained in combination

The His-tagged long-R^3-IGF-I was confiscated together with an injection vial of erythropoietin, two vials labelled as growth hormone, and an injection vial containing Hexarelin (Table 1, 1). This combination of substances may also be interesting for doping control authorities and may mask the use of specific drugs. While erythropoietin and hexarelin were found in the vials as indicated on their labels, no prohibited substances were detected in the growth hormone vials even after comprehensive screening, which further substantiates the fact that they were from a dubious source. Nevertheless, the intended combination of hexarelin, a growth-hormone-releasing peptide, and human growth hormone may circumvent the detection of growth hormone misuse by applying the currently used doping control methods. The primary test is based on two different assays with two different antibodies. One antibody detects preferentially the heterogeneous, endogenous growth hormone variants; the other antibody binds mainly the homogeneous 22 kDa recombinant growth hormone isoform. The ratio of the assays is used to evaluate a sample according to cut-off values.[32–33] If a growth-hormone-releasing peptide increases the endogenous release by activating the ghrelin receptor (which is independent of the growth hormone feedback system), an increase in the recombinant protein would be 'diluted' and could probably not be detected by methods used currently.

Other products

Products 3–5 from Table 1 were without label or of Chinese origin. One product was labelled Geotropin and contained recombinant growth hormone as the name already indicates (Table 1, 5) and product 3b contained human chorionic gonadotropin.

In products 3a and 4, which were both not labelled and were confiscated by police and customs, long-R^3-IGF-I (4) or at least peptides of long-R^3-IGF-I (3a) were found. All products containing an IGF-I analogue contained long-R^3-IGF-I and the number of products containing long-R^3-IGF-I indicates a very frequent use for performance enhancement (3 out of 11 confiscated products).

Conclusion

The products analyzed during 2009 show that black market products nowadays also include different peptide hormone-derived products rather than steroid hormone preparations only. From the confiscated products, only 4 out of 11 contained the substance and amount declared on their label, and long-R^3-IGF-I and human growth hormone were the proteins detected (or at least labelled) most frequently (three products each), which may indicate that they are also ordered and used very often. In contrast, the nutritional supplements containing GHRP-2 as well as the glass bottle with S-4 were labelled with the xenobiotic ingredients, although none of them is approved as a regular therapeutic agent yet. This summary gives an indication of products and

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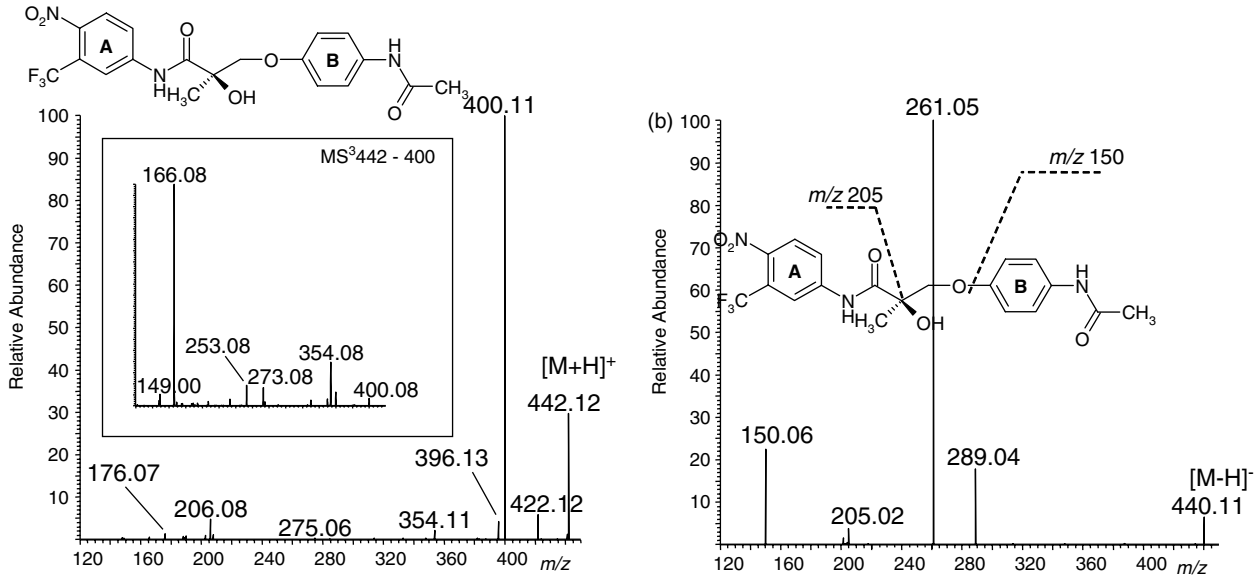

Figure 3. Product ion mass spectrum of S-4 in positive and negative mode with fragments clearly identifying Andarine.

combinations of compounds potentially misused in elite and amateur sport and stresses one more time the fact that the use of black market products implies a considerable health risk as a great share of the products do not (or not only) contain the substances indicated on the label.

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References

[1] W. Kondro, *Lancet* **2003**, *362*, 1466.
[2] D. H. Catlin, M. H. Sekera, B. D. Ahrens, B. Starcevic, Y. C. Chang, C. K. Hatton, *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1245.
[3] M. Thevis, U. Bommerich, G. Opfermann, W. Schänzer, *J. Mass Spectrom.* **2005**, *40*, 494.
[4] F. Fotiou, S. Aravind, P. P. Wang, O. Nerapusee, *Clin. Ther.* **2009**, *31*, 336.
[5] H. Geyer, M. K. Parr, K. Koehler, U. Mareck, W. Schänzer, M. Thevis, *J. Mass Spectrom.* **2008**, *43*, 892.
[6] M. Thevis, Y. Schrader, A. Thomas, G. Sigmund, H. Geyer, W. Schänzer, *J. Anal. Toxicol.* **2008**, *32*, 232.
[7] H. Geyer, U. Mareck-Engelke, W. Schänzer. **1998**, In W. Schänzer, H. Geyer, A. Gotzmann, Mareck-Engelke (eds), *Recent Advances in Doping Analysis*. Sport & Buch Strauß: Cologne, Vol. 6, pp. 421.
[8] F. Musshoff, T. Daldrup, M. Ritsch, *Arch. Kriminol.* **1997**, *199*, 152.
[9] M. R. Graham, P. Ryan, J. S. Baker, B. Davies, N-E Thomas, S. M. Cooper, P. Evans, S. Easmon, C. J. Walker, D. Cowan, A. Kicman, *Drug Testing and Analysis* **2009**, *1*, 135.
[10] M. Kohler, A. Thomas, K. Walpurgis, W. Schänzer, M. Thevis, *Growth Horm IGF Res* **2010**, DOI: 10.1016/j.ghir.2010.07.001.
[11] S. E. Bastian, P. E. Walton, J. C. Wallace, F. J. Ballard, *J. Endocrinol.* **1993**, *138*, 327.
[12] F. J. Ballard, S. E. Knowles, P. E. Walton, K. Edson, P. C. Owens, M. A. Mohler, B. L. Ferraiolo, *J. Endocrinol.* **1991**, *128*, 197.
[13] M. Bredehöft, W. Schänzer, M. Thevis, *Rapid Commun. Mass Spectrom.* **2008**, *22*, 477.
[14] D. S. Waugh, *Trends Biotechnol.* **2005**, *23*, 316.
[15] K. Terpe, *Appl Microbiol Biotechnol.* **2003**, *60*, 523.
[16] M. Uhlen, *Biotechniques* **2008**, *44*, 649.
[17] J. Argente, L. M. Garcia-Segura, J. Pozo, J. A. Chowen, *Horm. Res.* **1996**, *46*, 155.
[18] C. Pihoker, R. Middleton, G. A. Reynolds, C. Y. Bowers, T. M. Badger, *J. Clin. Endocrinol. Metab.* **1995**, *80*, 2987.
[19] M. Thevis, W. Schänzer, *Mini Rev. Med. Chem.* **2007**, *7*, 531.
[20] J. D. Veldhuis, D. M. Keenan, J. N. Bailey, J. M. Miles, C. Y. Bowers, *Eur. J. Endocrinol.* **2009**, *161*, 293.
[21] A. Thomas, M. Kohler, J. Mester, H. Geyer, W. Schänzer, M. Petrou, M. Thevis, *Drug Test. Analysis* **2010**, *2*, 144.
[22] V. Mericq, F. Cassorla, C. Y. Bowers, A. Avila, B. Gonen, G. R. Merriam, *J. Pediatr. Endocrinol. Metab.* **2003**, *16*, 981.
[23] A. Thomas, S. Guddat, M. Kohler, O. Krug, W. Schänzer, M. Petrou, M. Thevis, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1124.
[24] M. Okano, M. Sato, A. Ikekita, S. Kageyama, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2046.
[25] M. L. Mohler, C. E. Bohl, A. Jones, C. C. Coss, R. Narayanan, Y. He, D. J. Hwang, J. T. Dalton, D. D. Miller, *J. Med. Chem.* **2009**, *52*, 3597.
[26] M. Thevis, M. Kamber, W. Schänzer, *Rapid Commun. Mass Spectrom.* **2006**, *20*, 870.
[27] M. Thevis, M. Kohler, J. Maurer, N. Schlörer, M. Kamber, W. Schänzer, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3477.
[28] M. Thevis, M. Kohler, N. Schlörer, G. Fussholler, W. Schänzer, *Eur. J. Mass Spectrom. (Chichester, Eng)* **2008**, *14*, 153.
[29] M. Thevis, M. Kohler, N. Schlörer, M. Kamber, A. Kuhn, M. W. Linscheid, W. Schänzer, *J. Mass Spectrom.* **2008**, *43*, 639.
[30] M. Thevis, M. Kohler, A. Thomas, J. Maurer, N. Schlörer, M. Kamber, W. Schänzer, *Anal. Bioanal. Chem.* **2008**, *391*, 251.
[31] M. Thevis, H. Geyer, M. Kamber, W. Schänzer, *Drug Test. Analysis* **2009**, *1*, 387.
[32] M. Bidlingmaier, J. Suhr, A. Ernst, Z. Wu, A. Keller, C. J. Strasburger, A. Bergmann, *Clin. Chem.* **2009**, *55*, 445.
[33] M. Bidlingmaier, Z. Wu, C. J. Strasburger, *Baillieres Best Pract. Res. Clin. Endocrinol. Metab.* **2000**, *14*, 99.

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