

# Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement

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Black market products of a pharmaceutical nature and nutritional supplements have received substantial and increasing attention because of potential performance enhancement in elite and non-professional sports. In addition, improved general health is claimed for non-competing individuals. The risks and foreseeable dangers of the uncontrolled use of highly potent and non-approved pharmaceutical compounds in healthy individuals are of considerable concern. In the present case report, the emerging drug candidate GHRP-2 with verified growth-hormone-releasing properties was identified and quantified in tablets offered as an over-the-counter nutritional supplement. The impact of this orally active peptide on the hGH/IGF-axis has been established for several years and its illicit use in elite sports has been assumed. As a releasing factor for hGH, GHRP-2 belongs to the list of substances prohibited by the World Anti-Doping Agency (WADA). Unfortunately, to date there is no routinely performed assay for the determination of these peptides potentially occurring in biological fluids of competing athletes, but the present data will facilitate the implementation by providing principle analytical information on liquid chromatographic and mass spectrometric behaviour. Qualitative identification of the target analyte after extraction from the tablet matrix was performed by high resolution/high accuracy mass spectrometry after liquid chromatographic separation under consideration of the accurate masses and the ratios of the protonated molecules and their fragment ions derived from their collisionally induced dissociation. Quantitative results were obtained by means of liquid chromatography coupled to a triple quadrupole mass spectrometer and linear regression using an external calibration curve (with GHRP-2 reference compound) adjusted via internal standard (Hexarelin). Hereby, the content of GHRP-2 was determined with approximately 50 µg per tablet. Copyright © 2010 John Wiley & Sons, Ltd.

## Introduction

Boosting the endogenous production of human growth hormone (hGH) is desired by cheating athletes to enhance performance in elite as well as in non-professional sport, although the performance enhancing properties of hGH are still under discussion.<sup>[1]</sup> Endogenously, hGH is released in pulses from the pituitary by the growth-hormone-releasing hormone (GHRH) that is produced in the hypothalamus. As synthetic releasing factor analogues of this hormone, various penta-, hexa- or heptapeptides were found to possess hGH-releasing properties and, hence, they are prohibited in elite sports according to the list of substances prohibited by the World Anti-Doping Agency (ADA).<sup>[2]</sup> Due to their small size (<1000 Da), these peptides are bio-available after intravenous, subcutaneous, intranasal, buccal and also oral (iv, sc, in, b, po) administration and increase the amount of hGH and insulin-like growth factor-1 (IGF-1) in the treated individuals independently from gender but clearly age-related.<sup>[3–6]</sup> Known and well-investigated species of GHRPs are: GHRP-1, GHRP-2, GHRP-6, Ipamorelin and Hexarelin.<sup>[7]</sup> The amino acid sequences for these most promising candidates are illustrated in Table 1. The common part consisting of the amidated C-terminus ending with -Phe-Lys-NH<sub>2</sub> presumably represents the bioactive site of the peptides. In order to prove this, theories on structural pharmacophores based on molecular modelling and computational design were described in former studies.<sup>[4,7,8]</sup> In contrast to GHRH, the releasing peptides deemed to follow a counteract of somatostatinergic activity at hypothalamic as well as at pituitary level, but the complete pathways are still not entirely understood.<sup>[4,8]</sup> However, the administration (iv, sc, in, b or po) of

GHRPs in humans as well as in animals has been demonstrated to significantly increase the plasma level of hGH and, thus, induce all desired effects of this potentially performance-enhancing compound. It was clearly evidenced that GHRPs bind specifically on the ghrelin-receptor (different from GHRH-receptors) that is present in various tissues but mainly in the hypothalamus and the pituitary gland.<sup>[4,9]</sup> Among the presented peptides, GHRP-2 (Pralmorelin, GPA 748, growth-hormone-releasing peptide 2, KP-102 D, KP-102 LN, KP-102D or KP-102LN) is deemed to hold an exceptional position due to its potency and aberrant impact on bioactive processes.<sup>[9–11]</sup> The hexapeptide was discovered in 1993 and its effects are described to be synergistic with GHRH and, in addition, the somatostatin-induced suppression of hGH-secretion has been circumvented.<sup>[8]</sup> Hence, it is not surprising that this distinct peptide was discovered in a black market product, advertised to possess growth promoting potential. The prod-

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**Table 1.** Growth-hormone-releasing peptides with their amino acid sequence, elemental composition, monoisotopic masses and year of discovery. (Non-standard abbreviations: Nal = naphthylalanine, Mrp = 2-methyltryptophane, Aib = aminoisobutyric acid)

name	amino acid sequence	elemental composition	monoisotopic mass [Da]	discovered
GHRP-2	(D-Ala)-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-NH <sub>2</sub>	C <sub>45</sub> H <sub>55</sub> N <sub>9</sub> O <sub>6</sub>	817.4270	1993
GHRP-1	Ala-His-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-NH <sub>2</sub>	C <sub>51</sub> H <sub>62</sub> N <sub>12</sub> O <sub>7</sub>	954.4859	1991
GHRP-6	His-(D-Trp)-Ala-Trp-(D-Phe)-Lys-NH <sub>2</sub>	C <sub>46</sub> H <sub>56</sub> N <sub>12</sub> O <sub>6</sub>	872.4440	1984
Hexarelin	His-(D-Mrp)-Ala-Trp-(D-Phe)-Lys-NH <sub>2</sub>	C <sub>47</sub> H <sub>58</sub> N <sub>12</sub> O <sub>6</sub>	886.4597	1992
Ipamorelin	Aib-His-(D-2-Nal)-(D-Phe)-Lys-NH <sub>2</sub>	C <sub>38</sub> H <sub>49</sub> N <sub>9</sub> O <sub>5</sub>	711.3851	1996

uct was provided as blistered tablet form (90 tablets) for oral intake.

In this case report, a qualitative identification and quantification of the approximate content of GHRP-2 in tablets offered as nutritional supplements by means of liquid chromatography coupled to high resolution/high accuracy mass spectrometry is described.

## Experimental

### Reference compounds and nutritional supplement

Reference substances of GHRP-2 and Hexarelin, used as internal standard (ISTD), were obtained from Purepeptides (San Diego, Cam USA). The purity and identity of GHRP-2 was confirmed by means of liquid chromatography coupled to high resolution/high accuracy mass spectrometry (data not shown).

The black market product of GHRP-2, declared as a nutritional supplement, was purchased over-the-counter in Cyprus (in September 2009) as blistered tablets (3 × 30 pieces), with an expiry date of October 2011. The tablets had a weight of approximately 2 g and were coated with red gloss. As the active ingredient, GHRP-2 with the following (obviously wrong) amino acid sequence was declared (His-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-OCH<sub>3</sub>) without indicating the amount of drug per tablet. The recommended daily dose of the product was 3 tablets, administered 30 min before going to bed.

### Sample preparation

For proper analysis, one tablet was ground with a pestle and the obtained powder was sonicated for 1 h in 10 mL of a mixture of acetonitrile/water (50/50, v : v). One hundred μL of the supernatant was diluted with 900 μL of acetic acid (2%), fortified with internal standard (0.2 ng) and subjected to liquid chromatography – mass spectrometry (LC-MS) analysis. All dilutions were made in 1.5 mL polypropylene tubes.

### LC-MS

Qualitative identification of GHRP-2 was performed by means of high resolution/high accuracy mass spectrometry with an Exactive mass spectrometer (Thermo, Bremen, Germany) after liquid chromatographic separation using an Accela UPLC (Thermo, Bremen, Germany) equipped with a Zorbax SB300 analytical column, 0.3 × 50 mm, 5 μm particle size (Agilent, Waldbronn, Germany) and a guard column Zorbax SB300, 1 × 17 mm, 5 μm particle size. LC was performed with a gradient program (Start: 95% A, in 10 min to 10% A, hold 1 min, re-equilibrate

for 10 min at 95%A, flow 25 μL/min) using formic acid (0.2%) as aqueous solvent (A) and acetonitrile as organic solvent (B). The mass spectrometer was equipped with a Nanomate (Advion, Ithaca, NY, USA) nano-electrospray ion (NSI) source in positive LC-coupling mode. The LC-split conditions were set to an approximate flow to the NSI-chip of 500 nL/min with 1.5 kV ionisation voltage and a capillary temperature of 175 °C. Three experiments including MS and higher-collision-induced dissociation (HCD)-MS at two different energies (20 and 50 eV) were acquired in the mass analyzer. The resolution power was set to 50 000 FWHM and reliable mass accuracies were ensured by mass calibration using the manufacturer's calibration kit.

Quantitative analysis was performed on an ABI 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an Agilent 1100 liquid chromatograph (Waldbronn, Germany) by MRM experiments (*m/z* 409.7/170 for GHRP-2 and 444.0/338 for ISTD) in positive ionisation mode with a collision offset voltage of 35 V and a declustering potential of 20 V. The analytical column was a Zorbax 300SB, 2.1 × 50 mm, 3.5 μm particle size (Agilent, Palo Alto, CA, USA) and the solvents consisted of A: 0.1% acetic acid and B: acetonitril/0.1% acetic acid (80/20, v : v). The gradient started at 90% A and decreased to 20% A in 8 min, followed by an re-equilibration for 7 min at starting conditions. The flow rate was set to 300 μL/min and the runtime was 15 min.

### Calibration curve

For quantitative analysis, the approximate content of one tablet was determined by linear regression with a calibration curve range from 0.1 to 1.0 ng/mL (0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1.0 ng/mL) using the GHRP-2 reference compound. Here, Hexarelin with 0.2 ng/mL was used as and ISTD and quantification was calculated via non-weighting peak area ratios.

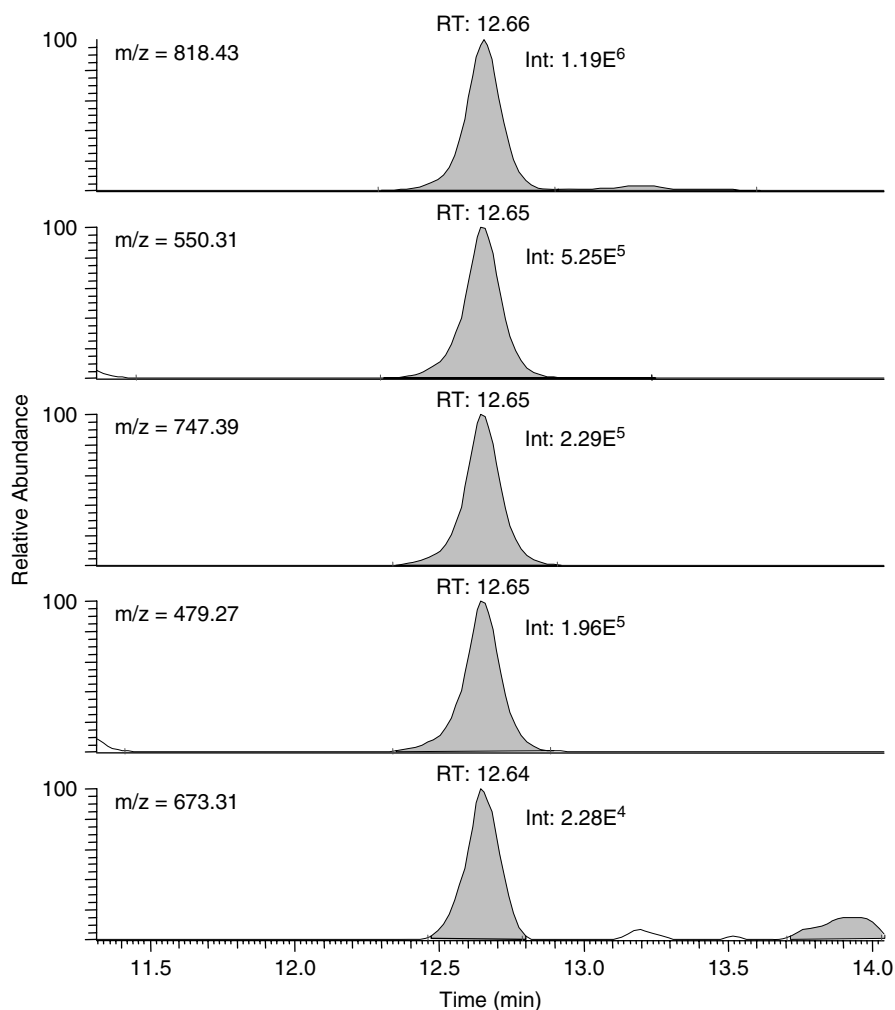
## Results

### Qualitative identification

The identity of GHRP-2 was confirmed by the determination of the accurate masses (<1.5 ppm) of the protonated and diprotonated molecules (at *m/z* 818.435 and 409.721), the ratios of their derived HCD-fragments and the retention times obtained from analysis of the extracted tablets and the reference compound. The main results with measured masses, errors and retention times are summarized in Table 2. This strategy follows the main issues of the commonly used procedure and requirements from

**Table 2.** Main mass spectrometric results for the qualitative identification of GHRP-2 from extracted tablets illustrated as experimental masses, errors, product ion identification with elemental composition and comparison of relative peak areas ratios and retention times obtained from the extracted tablet and the reference compound of GHRP-2

masses (exp.) [ <i>m/z</i> ]	error [ppm]	ion	elemental composition	ion transition [ <i>m/z</i> ]	reference		tablet	
					ret. time [min]	rel. area ratio	ret. time [min]	rel. area ratio
818.4347	-0.12	[M + H] <sup>+</sup>	C <sub>45</sub> H <sub>56</sub> O <sub>6</sub> N <sub>9</sub>	818.434	12.65	100%	12.66	100%
409.7213	0.66	[M + 2H] <sup>2+</sup>	C <sub>45</sub> H <sub>57</sub> O <sub>6</sub> N <sub>9</sub>	-	-	-	-	-
747.3973	-0.58	y <sub>5</sub> <sup>+</sup>	C <sub>42</sub> H <sub>51</sub> O <sub>5</sub> N <sub>8</sub>	747.397	12.66	21.5%	12.65	19.4%
673.3124	-1.26	b <sub>5</sub> <sup>+</sup>	C <sub>39</sub> H <sub>41</sub> O <sub>5</sub> N <sub>6</sub>	673.312	12.66	1.7%	12.65	1.8%
550.3134	-0.38	y <sub>4</sub> <sup>+</sup>	C <sub>29</sub> H <sub>40</sub> O <sub>4</sub> N <sub>7</sub>	550.313	12.66	49.4%	12.65	46.2%
479.2768	0.64	y <sub>3</sub> <sup>+</sup>	C <sub>26</sub> H <sub>35</sub> O <sub>3</sub> N <sub>6</sub>	479.277	12.65	18.4%	12.64	16.7%

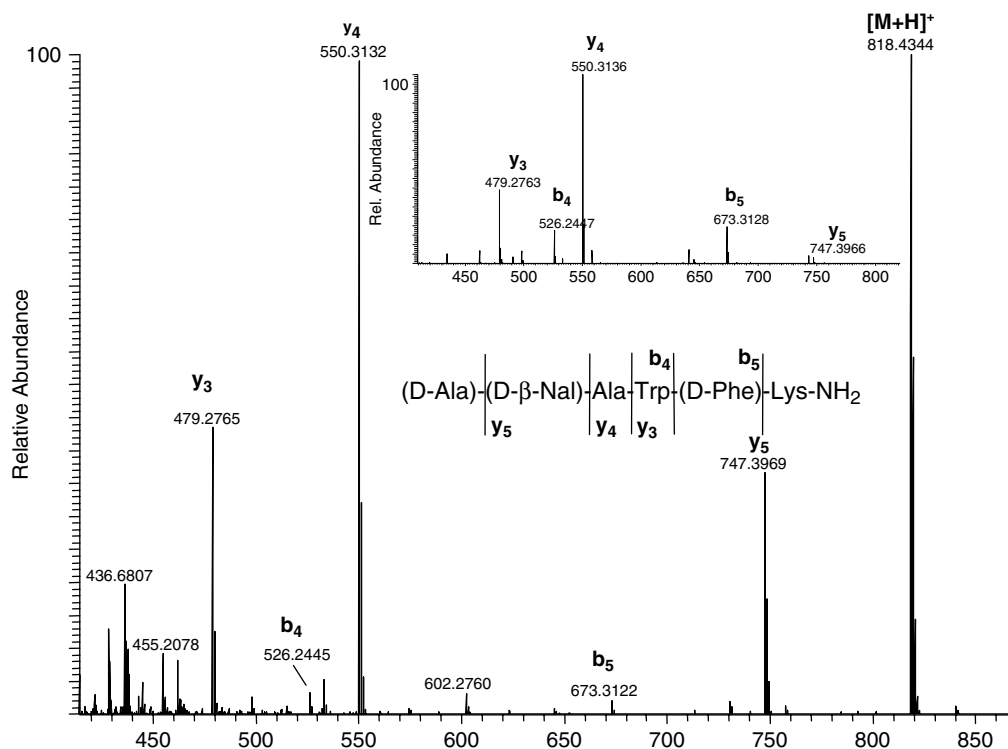
**Figure 1.** Extracted ion chromatograms with diagnostic ion traces at *m/z* 550.313 (y<sub>4</sub>), 747.397 (y<sub>5</sub>), 479.277 (y<sub>3</sub>) and 673.312 (b<sub>5</sub>) with abundant signals at 12.65 min from a liquid-extracted tablet. Intensities are referring to peak heights.

WADA and recently published recommendations for peptides <8 kD.<sup>[12,13]</sup>

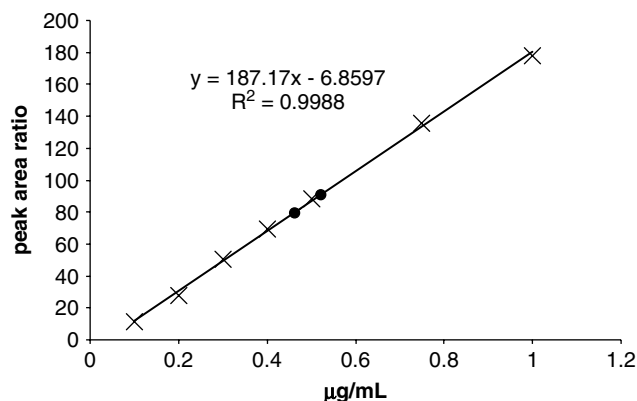
Fragments were identified as common HCD-generated y and b ions deriving from the peptide core of the target molecule.<sup>[14]</sup> The most abundant fragment ions at *m/z* 550.313 (y<sub>4</sub>), 747.397 (y<sub>5</sub>), 673.312 (b<sub>5</sub>) and 479.277 (y<sub>3</sub>) from the peak at 12.66 min were chosen for identification. The chromatograms with the respective mass spectrum from a properly extracted and diluted tablet are shown in Figures 1 and 2.

#### Quantitative determination

Quantification of the active peptide in the tablet after external calibration with ISTD yielded in an approximate mean content of 49.9 µg/tablet. Main regression characteristics were provided with a slope and intercept of the calibration curve  $y = 187.17x - 6.86$  and a coefficient of determination of  $R^2 = 0.9988$  (Figure 3). In order to reach more reliable results, determination was performed with two independent extractions from two different



**Figure 2.** HCD-mass spectrum recorded at a collision energy of 20 eV from an extracted tablet yielding diagnostic product ions deriving from the peptide backbone of GHRP-2. The inset shows the mass spectrum from the corresponding product ion experiment of the doubly charged precursor at  $m/z$  409.7 measured on a LTQ Orbitrap mass analyzer, where the origin of the diagnostic product ions was confirmed.



**Figure 3.** Quantification of GHRP-2 from extracted tablets by means of an external calibration and internal standard. Calibration points are illustrated as  $\times$  and results for the two-fold determination from different tablets are shown as  $\bullet$ .

tablets ( $n=2$ ) and the relative standard deviation of two parallel determinations was 8.4%.

## Discussion

As a drug, GHRP-2 has proceeded to phase-II stage in clinical trials and, thus, is still a non-approved pharmaceutical agent.<sup>[15]</sup> It was originally developed to enhance the hGH-release from the pituitary and to treat short-stature or distinct kinds of hGH deficiency in children or young adults. Possible side effects, exact dose-effect studies and other important parameters are not entirely

evaluated yet. Nutritional supplements must not contain any bioactive compound to treat, diagnose or cure diseases.<sup>[16]</sup> Despite these facts, GHRP-2 is offered as over-the-counter nutritional supplement tablets (without prescription), which illustrates the danger of potential misuse, not only as a matter of preventive doping research, but also for general health protection concerns. This is further supported by considering the obviously incorrectly labelled amino acid sequence on the package (C-terminus  $-Lys-OCH_3$  instead of  $-Lys-NH_2$ ) and the lack of quantitative information about the active drug per tablet.

## Conclusion

The presented case report demonstrates the urgency of flexible analytics in doping controls. Although to date no positive doping cases with GHRP-2 were reported, the fact that the bioactive compound is available as a nutritional supplement, analytical findings in routinely analyzed plasma or urine samples from elite sportsmen are possible. Noteworthy, a non-targeted analytical approach by means of high resolution/high accuracy mass spectrometry has recently been developed and proves the possibility of detecting GHRP-2 in plasma samples down to low ng/mL range.<sup>[17]</sup> Renal clearance and urinary concentrations after administration are still unclear and will be evaluated in further studies. First suggestions for the estimated plasma concentrations are presumably in low ng/mL range considering a bioavailability of 0.3 to 1% of the orally applied peptide.<sup>[6,8]</sup> Considering that the maximal effective dose is described with  $1 \mu\text{g}/\text{kg}$  (iv), a recommended oral intake of 3 tablets yield for a normal subject ( $\sim 70 \text{ kg}$ ) in peak plasma levels ( $C_{\text{max}}$ ) approximately 2% of this

maximal dose and, thus, potential plasma or urinary concentrations after application will be presumably in sub-ng range.

### Acknowledgements

The study was carried out with the support of the Manfred Donike Institute for Doping Analysis, Cologne, Germany; the Federal Ministry of the Interior of the Federal Republic of Germany; and the Cyprus Anti-Doping Authority.

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