Journal of Chromatography
Separation Techniques

Ferro et al., J Chromatogr Sep Tech 2017, 8:1
DOI: 10.4172/2157-7064.1000351

Research Article Open Access

Detection of Growth Hormone Releasing Peptides in Serum by a Competitive Receptor Binding Assay

Ferro P[1]*, Krotov G[2], Zvereva I[2], Pérez-Mana[3,4], Mateus JA[3] and Segura J[1,5]

[1]*Bioanalysis Research Group, Neuroscience Research Program, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain*
[2]*Anti-Doping Centre, Moscow, Russia*
[3]*Integrative Pharmacology and Systems Neuroscience Research Group, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain*
[4]*Department of Pharmacology, Therapeutics and Toxicology, Autonomous University of Barcelona, Cerdanyola, Spain*
[5]*Department of Experimental and Health Sciences, Pompeu Fabra University (UPF), Barcelona Biomedical Research Park, Barcelona, Spain*

Abstract

The use of small peptides for medical purposes has gained great importance in the last decade. Among these molecules are the growth hormone releasing peptides (GHRPs). These substances are highly sought by cheating athletes because of their ergogenic effects. Thus, GHRPs are listed in the World Antidoping Agency Prohibited List and several methods have been developed for their detection in urine samples. Serum is another useful matrix frequently used for anti-doping control. An experimental adaptation of a competitive methodology for GHRPs wide-group detection, previously developed for urine, is reported here using a serum matrix instead. The modifications have been mainly addressed the need to remove endogenous ghrelin (always present in serum) to avoid interference in the competitive binding assay. The modified test was first evaluated using serum spiked with different pure GHRPs (i.e., GHRP-2, GHRP-6 and Hexarelin), then using serum samples from pilot studies in which healthy subjects were treated intravenously with GHRP-2 and intranasally with GHRP-2 and GHRP-6. The results showed the reliability of the modified methodology for the detection of GHRPs in a serum matrix.

Keywords: Growth hormone secretagogue; Ghrelin; Radiocompetition assay; Doping substances; Doping abuse

Introduction

During the past decade, small molecules have been target of a wide range of applications in medicine and biotechnology. Therapeutic peptide research is also currently experiencing a renaissance for commercial interest [1], among others reasons. Growth hormone secretagogues (GHSs) are active small molecules with medical and sport performance applications. GHSs constitute a complex heterogeneous family of compounds that keeps growing in number and can be roughly divided into two main groups: 1) growth hormone releasing hormone and analogs such as Sermorelin and CJC-1295, and 2) growth hormone releasing peptides (GHRPs), which show a high stimulatory activity on growth hormone (GH) release [2].

GHRPs stimulate GH release and have been regarded as an alternative to support diagnostics and treat diseases related to GH deficiency [3,4]. Their effects have been described *in vivo* and *in vitro*, in both animals and humans, using various doses and administration routes [5-10]. However, only the peptide GHRP-2 (also known as pralmorelin) has been clinically tested and approved in Japan for diagnostic purposes [11], while other GHRPs have not passed clinical trials or are still in different stages of development.

One of the interesting features of GHRPs is the fact that they can be active by several administration routes, including oral administration. Besides, their ergogenic ability has been reported, so they are becoming very appreciated as substances of abuse by cheating athletes that seek new alternatives to traditional doping [12,13]. In fact, several of these GHRPs are available in the black market and can be obtained easily on the Internet [14,15]. Consequently, the World Anti-Doping Agency (WADA) incorporated GHRPs in its Prohibited List of doping substances since 2013, and must be detectable by anti-doping laboratories [16].

Anti-doping detection methods based in liquid chromatography–mass spectrometry (LC-MS) have been developed in recent years using urine as main matrix [17-21]. These methods are focused especially on those GHSs with known structure and metabolism which could be used more frequently in sport abuse. Nonetheless, considering that these molecules belong to a pharmacological family, the development of wide scope detection methods for all GHSs known as well as unknown, could be a highly desirable resource [22-24]. Such universal detection methods could be based on a common feature, e.g., their interaction with the GHS-R1a receptor, for which the endogenous natural ligand is ghrelin, a 3-octanoylated 28 amino acid peptide [25].

Traditionally, urine was the sample of choice for the screening and identification of unknown drugs due to high concentration of drugs in urine. Therefore, this matrix has been used or GHSs (no: or GHSs) detection in athlete samples with anti-doping purposes [21]; although the use of another matrices, as serum, could be highly desirable. However, the GHRPs studies performed so far with serum samples have been carried out only in a few clinical trials with healthy males and children administered with GHRP-2 and GHRP-6 respectively [26,27]. In these clinical trials a pharmacokinetic study for both GHRPs was performed for a maximum time of 12 hours. For sample analysis, a specific anti-GHRP-2 polyclonal antibody was used in a radioimmunoassay to detect GHRP-2 [26] and a LC-MS method was developed to detect GHRP-6 [27].

***Corresponding author:** Ferro P, Bioanalysis Research Group, Neuroscience Research Program, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain, Tel: +34 933160470; E-mail: pedro.ferro@ibima.eu

Received February 02, 2017; **Accepted** February 15, 2017; **Published** February 21, 2017

Citation: Ferro P, Krotov G, Zvereva I, Pérez-Mana, Mateus JA, et al. (2017) Detection of Growth Hormone Releasing Peptides in Serum by a Competitive Receptor Binding Assay. J Chromatogr Sep Tech 8: 351. doi: 10.4172/2157-7064.1000351

Copyright: © 2017 Ferro P, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Here we present an experimental study which was built on the previous screening method described by Pinyot et al. and Ferro et al. in urine samples [22,28,29] to develop an alternative method that uses serum as a new matrix. The serum is a valuable matrix used for analysis of some doping substances e.g., CERA [30], human chorionic gonadotrophin [31], and growth hormone [32,33]. A GHRPs screening method for anti-doping control in serum samples would be highly desirable. However, serum contains a substantial amount of the natural ligand ghrelin, which was expected to interfere with the determination of GHRPs by competitive binding to the corresponding membrane receptor (GHS-R1a) in the assay. This work assessed this potential interference and implemented steps to remove it. The removal of natural ghrelin from serum by plate immunocapture appeared as a suitable alternative. The performance of this new protocol was assessed in serum samples from pilot studies in which healthy subjects were administered intravenously with a bolus of 100 µg of GHRP-2, intranasally with 200 µg of GHRP-2, intranasally with 200 µg of GHRP-6, or as untreated controls (n=4). Clear competitive signal of radioactive ligand displaced from the GHS-R1a receptor was verified in all administration studies.

Methods and materials

Chemicals

For *in vitro* studies, growth hormone-releasing peptide 2 (GHRP-2, pralmorelin) was kindly supplied by Dr. S. Kageyama (Tokyo Laboratory, Anti-Doping Center, Mitsubishi Chemical Medience, Japan) whereas GHRP-6 and Hexarelin were kindly supplied by Giampiero Muccioli (University of Torino, Italy). For the intravenous excretion studies, GHRP KAKEN100® (pralmorelin dihydrochloride, 100 µg/vial) was purchased from Kaken Pharmaceutical Co., Ltd. For intranasal studies, GHRP-2 and GHRP-6 were synthesized by Thermo Fisher Scientific GmbH (Ulm, Germany). Radiolabeled (125I-His9) ghrelin (no: Rdiolabeled ghrelin (a125-His9) ghrelin) was purchased from Perkin Elmer (Waltham, MA, USA). All other chemicals were of the highest grade commercially available.

Cell culture, membrane preparations and competition binding assay

Cell culture, membrane preparations and competition binding assay parameters have previously been published by Pinyot et al. [22] and Ferro et al. [28] Data were analysed and plotted with GraphPad Prism 5 (San Diego, CA, USA) or Excel software. Statistical analysis was performed using Excel and SPSS programs. To contrast independent continuous variables, Student's t-test was used. Null hypothesis was rejected for values of $p \leq 0.05$.

Reference samples

Two reference samples in binding buffer were included in each competition binding experiment, one as blank or negative control (containing cells and all reagents but not spiked with GHRPs) as the maximum possible binding (100% of relative specific binding, RSB) and a second sample or positive control, spiked with 7.5 µM GHRP-2, as the minimal possible specific binding (0% RSB). All sample binding values were calculated relative to these limits.

GHRPs treatment in human subjects

Intravenous GHRP-2: An injectable solution of GHRP KAKEN100® (10 µg/mL in saline), containing 100 µg pralmorelin dihydrochloride (GHRP-2), was administered intravenously after overnight fasting. The protocol was approved by the Clinical Research Ethical Committee from Parc de Salut Mar (CEIC-Parc de Salut Mar), Barcelona (n°2014/5760). Eight healthy caucasian male volunteers were administered with the drug and two control subjects were left untreated. The subjects were 20 to 30 years old, mean weight 72.9 ± 8.7 and body mass index (BMI): 21.5 ± 3.5. All subjects were informed in advance of the study details and signed the corresponding informed consent letter. The volunteers refrained from ingesting alcohol and any medicines and did not perform any exercise during the studies. Urine and serum samples were collected prior to administration of the drug and several times during the 24 h period after administration. All samples were stored at -20°C until analysis.

Intranasal GHRP-2 and GHRP-6: A single nasal dose of one of the peptides GHRP-2 or GHRP-6 (200 µg) was administered to four volunteers (two for GHRP-2 and two for GHRP-6) alongside with two control subjects without any administration) by the Anti-Doping Centre in Moscow. Serum samples from each volunteer were collected prior to drug administration and several times during the 24 h period after administration. Samples were stored at –20°C until analyses.

Analytical aspects

Serum: During method development, serum samples were subjected to different treatments. Additional details appear in the results section.

Thermic treatment: 150 µl of serum samples were incubated at 37°C and 50°C for 1,2 and 4 h in a thermomixer and evaluated by the competition binding assay (see above).

Incubation at different pHs: 150 µl of serum samples were treated to reach pH 7.4 or 9 and were incubated at 37°C and 50°C in a thermomixer for 1 h. After treatment samples were evaluated by the competition binding assay (see above).

Immunocapture: Antibody anti-ghrelin coated microplates (Cayman Chemicals, Ann Arbor, MI, USA) were washed 5 times with 300 µl of wash buffer (phosphate-buffered saline (PBS) and 0.05% Tween-20). 150 µl of serum samples were thawed, deposited in a well and incubated at 4°C under agitation (orbital shaker) for 1, 2, 4 h or overnight. Serum samples recovered from the wells were evaluated by the competition binding assay (see above).

According to these developments and the results obtained (see below), the final chosen methodology involved 4 hours incubation followed by a second incubation of the same serum in another well in the same conditions for 2 h. Eventually, samples were recovered again from the second well and 40 µl were evaluated in triplicate by the competitive binding assay (see above).

Urine: Urine samples were processed following the protocol established by Pinyot et al. [29]. Briefly, urine samples were thawed and centrifuged for 15 min at 3500g at 4°C. After desalting of 2.5 ml of urine by loading into a 3-ml Oasis HLB solid phase extraction (SPE) cartridge (Waters, Mildford, USA), the eluted sample obtained with methanol was dried in an N_2-evaporator TurboVap LV (Caliper, Hopkinton, USA). The dried residue was reconstituted in 1 ml fresh prepared binding buffer, sonicated for 5 min and GHRPs were purified from the sample by receptor affinity. Purified samples were reconstituted in 150 µl of binding buffer and analysed by the competitive binding assay (see above).

Results and Discussion

There were several important reasons for our interest in the

development of a novel detection method for GHRPs in serum: 1) the increased and frequent use by cheating athletes of these compounds, which can be easily purchased in the black market or via Internet, 2) the relative complexity of the method for urine (the protocol takes three days to be completed), and 3) the fact that blood samples can be used for the detection of some doping substances that cannot be detected in urine [33].

The previously described radio competition assay methodology to detect GHRPs in urine [22,28,29] was modified and simplified for the detection of these compounds in serum samples. As was expected, however, the direct application of the competitive assay on "unprocessed" serum samples (without any denaturing or immunocapture pretreatment) from 10 non-treated subjects showed an interfering effect. An RSB with mean and SD of 82.97% ± 3.68, indicated the presence of background amounts of receptor binding compound(s) (presumably ghrelin) when compared with negative reference sample in binding buffer (100% RSB). Notably, serum samples from volunteers 8, 9 and 10 had RSB values of 70.98 ± 6.23, 66.74 ± 1.37 and 68.07 ± 0.93%, respectively, indicating a high interference background. As a consequence, different variables had to be studied to remove the endogenous interfering substance(s) from the serum, as mentioned below.

Temperature and pH treatment

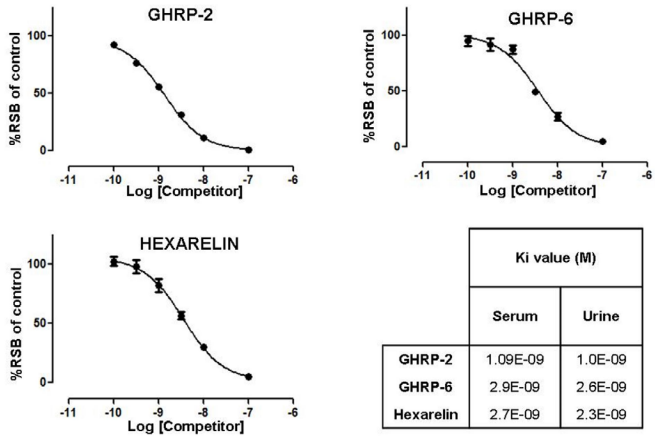
Ghrelin (together with non-acylated ghrelin) concentrations in plasma or serum have been found around 100–200 fmol/ml and their levels are increased before meals to 300 fmol/ml [34-36]. This could cause interference in the assay with serum samples and consequently generate However, it is well known that the ghrelin protein is sensitive to specific pH and temperature conditions [37,38], with a partial conversion from acylated ghrelin to des-acyl ghrelin. Des-acyl ghrelin has much lower capacity to interact with the GHS-R1a receptor. To assess whether the temperature and pH have effects on the assay regarding the reduction of endogenous ghrelin concentrations, serum samples were incubated at 37°C and 50°C for several hours (1, 2 and 4 h). Results are displayed in Table 1. It was found an increase in the RSB values in samples treated at 37°C and 50 °C after two hours of incubation. Higher effects were observed in samples incubated at 50°C for 4 hours, with 92.67 ± 1.29% RSB, as compared to 74.63 ± 1.26% RSB for the non-incubated serum. Unfortunately, samples from volunteers having a lower baseline RSB showed improvement with temperature treatment, but it was not the case in those with baseline RSB greater than 77%.

Regarding the experiments involving incubation steps at neutral and basic pHs (pH 7.4 and pH 9.0, respectively) and different temperatures (37°C and 50°C) for 1 h, the results did not show significant differences before and after incubation (Table 1), therefore a different alternative would be needed in order to remove the endogenous ghrelin.

Time (h)	pH			
	7.4		9	
	37°C	50°C	37°C	50°C
1	71.73 ± 9.91	73.48 ± 8.69	74.04 ± 4.16	74.04 ± 1.71
2	81.29 ± 1.33	87.61 ± 8.16	--	--
4	90.85 ± 2.09	94.68 ± 3.77	--	--
NI	74.63 ± 1.26			

NI: Non incubated.

Table 1: Temperature and pH treatment in serum samples.



	Ki value (M)	
	Serum	Urine
GHRP-2	1.09E-09	1.0E-09
GHRP-6	2.9E-09	2.6E-09
Hexarelin	2.7E-09	2.3E-09

All points in the figure are depicted with error bars that represent the standard errors of the mean. Ki values for urine are taken from reference Pinyot et al.

Figure 1: Competition curves, showing the effect of GHRP-2, GHRP-6 and Hexarelin (x axis) on the inhibition of (^{125}I-His9) ghrelin binding in serum [21].

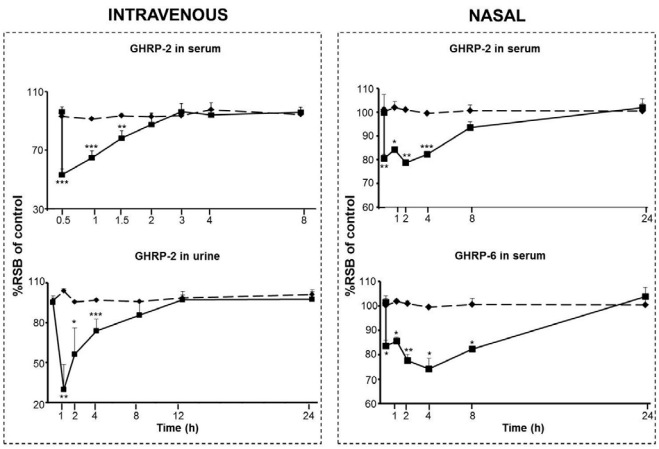


Figure 2: RSB profile in human samples after intravenous and intranasal administration of GHRPs.

Immunocapture

The most successful approach to remove endogenous ghrelin content in serum samples was based on antibody capture using microplates coated with anti-ghrelin antibodies. With this goal, 150 µl of serum were incubated in the antibody-coated wells for different time periods (1, 2, 4 h and overnight) and the serum recovered was assessed by radiocompetition assay. The results showed a RSB values of 86.71 ± 0.55%, 89.03 ± 0.68%, 92.67 ± 1.29% and 88.42 ± 5.03% for 1, 2, 4 h and overnight incubation, respectively, what indicated an increase ghrelin removal as compared to the non-incubated serum samples with mean RSB value of 74.33 ± 2.05%. However, a small background binding was still detected in the samples after a single immunocapture step.

Therefore, to remove all endogenous ghrelin, serum samples from 4 volunteers were subjected to two consecutive immunocapture steps: incubating first for 4 h in anti-ghrelin antibody-coated wells, and then incubating the recovered serum for 2 additional hours in distinct antibody-coated wells. After analysis by radiocompetitive assay, all samples showed RSB values close to 100%, with mean and SD between 96.02 ± 1.42% and 111.12 ± 1.74%, indicating the removal of most of

the interfering ghrelin.

Radiocompetition assay with different GHRPs

The assessment of the urine protocol with several different GHSs showed high robustness and sensitivity [22]. To optimize the radiocompetition assay to detect GHRPs in serum (ghrelin-depleted by immunocapture), the method was applied to serum samples spiked with different concentrations of three different GHRPs (GHRP-2, GHRP-6 and Hexarelin). The respective competition binding curves (Figure 1) showed serum Ki values similar to those obtained by Pinyot et al. for these GHRPs in urine samples [22]. These results confirmed that the newly implemented ghrelin immunocapture step in serum samples led to the development of a successful method for this matrix.

Detectability in serum and urine samples after GHRP-2 intravenous administration

The few alternative methods previously described to detect GHSs in serum samples are rather specific for one or few GHRPs based on radioimmunoassay [26] or LC-MS methods [27]. However, the new radiocompetition assay presented in this study could be the first method which can detect any known or unknown GHRP in this matrix. In order to verify the effectiveness and the detection time window of our protocol in serum samples, a pilot study in healthy male volunteers treated with GHRP-2 was performed. Serum and urine samples from a group of healthy male volunteers dosed intravenously with 100 µg of pralmorelin dihydrochloride (GHRP-2) were collected at different time points (0, 0.5, 1, 1.5, 2, 3, 4 and 8 h for serum and 0, 1, 2, 4, 6, 12, and 24 h for urine) post-administration. Samples from eight treated subjects and two untreated controls were assessed by the radiocompetition assay.

The results, presented in Figure 2-left, showed that all serum and urine samples collected immediately (and up to 2 h for serum and 6 h for urine) after the intravenous GHS injection displaced (125I-His9) ghrelin from the GHS-R1a receptor to a large extent. The basal state was subsequently recovered and remained unaltered until at least 24 h post-administration. The detection time window appeared shorter in the serum as the drugs can be detected for 2 hours after administration versus 6 hours in urine samples [29], although these data are similar to that obtained previously from GHRP-2 and GHRP-6 in serum samples with other methods [26,27].

GHRP-2 and GHRP-6 detection in serum samples after intranasal administration

Serum samples from healthy volunteers administrated with an intranasal dose of GHRP-2 or GHRP-6 were collected at different time points (0, 0.5, 1, 2, 4, 8 and 24 h for serum) post-administration. Samples from four treated subjects (two for GHRP-2 and two for GHRP-6) and two controls were assessed by the radiocompetition assay. The results, presented in Figure 2-right, showed that all GHRP-2 and GHRP-6 serum samples collected immediately and up to 8 h after intranasal administration, displaced (125I-His9) ghrelin from the GHS-R1a receptor to a large extent. The basal state was subsequently recovered and remained unaltered until at least 24 h post-administration.

The detection time window for GHRP-2 appeared shorter in serum as the drug can be detected for 8 h after administration in serum versus 18 h in urine samples [39]. For GHRP-6, the detection windows obtained here in serum and previously in urine [39] samples were very similar, so it also would be very interesting to study other GHRPs

Conclusion

A new wide-group screening method to detect GHRPs in serum samples has been developed and tested in human samples after administration of various GHRPs through different administration routes. This method showed a similar sensitivity and detection time window in serum in comparison with other protocols specified for a unique GHRP after intravenously administration. The detection window after intranasal administration of some GHRPs might be even similar regardless of the matrix used for the analysis (i.e., urine or serum). The approach described here has the additional advantages of being completed in a short time (only one day versus three days in the case of urine), easy to perform (needing only an initial immunocapture step with anti-ghrelin antibody to remove endogenous ghrelin) and requires a small volume of sample (40 µl), which is easy to obtain from any venipuncture protocol.

Acknowledgments

The authors thank Professor F. Casanueva and Professor R. Smith for HEK293 cells expressing GHS-R1a; Professor Rafael de la Torre and Professor Magi Farré for sharing the GHRP-2 study samples; and the Clinical Research Unit staff (Esther Menoyo, Marcos Wenceslao Pestarino, Cristina Llop, Clara Gibert, Soraya Martin and Marta Pérez) for their assistance. Clara Pérez-Mañá was funded by a Juan Rodes fellowship (ISC-III, JR15/00005).

References

1. Fosgerau K, Hoffmann T (2015) Peptide therapeutics: current status and future directions. Drug Discov Today 20: 122-128.
2. Arvat E, Di Vito L, Maccagno B, Broglio F, Boghen MF, et al. (1997) Effects of GHRP-2 and hexarelin, two synthetic GH- releasing peptides, on GH, prolactin, ACTH and cortisol levels in man. Comparison with the effects of GHRH, TRH and hCRH. Peptides 18: 885-891.
3. Bowers CY, Momany FA, Reynolds GA, Hong A (1984) On the in vitro and *in vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology 114: 1537-1545.
4. Bowers CY, Alster DK, Frentz JM (1992) The growth hormone-releasing activity of a synthetic hexapeptide in normal men and short statured children after oral administration. J Clin Endocrinol Metab 74: 292-298.
5. Esposito S, Deventer K, Geldof L, Peter VE (2015) In vitro models for metabolic studies of small peptide hormones in sport drug testing. J Pept Sci 21: 1-9.
6. Johansen PB (1998) Pharmacokinetic evaluation of ipamorelin and other peptidyl growth hormone secretagogues with emphasis on nasal absorption. Xenobiotica 28: 1083-1092.
7. Pihoker C, Badger TM, Reynolds GA, Bowers CY (1997)Treatment effects of intranasal growth hormone releasing peptide-2 in children with short stature. J Endocrinol 155: 79-86.
8. Hayashi S, Okimura Y, Yagi H, Toshiyuki U, Yashuhiro T (1991) Intranasal administration of His-D-Trp-Ala-Trp-D-Phe-LysNH2 (growth hormone releasing peptide) increased plasma growth hormone and insulin-like growth factor-I levels in normal men. Endocrinol Jpn 38: 15-21.
9. Ghigo E, Arvat E, Gianotti L, Imbimbo BP, Lenaerts V (1994) Growth hormone-releasing activity of hexarelin, a new synthetic hexapeptide, after intravenous, subcutaneous, intranasal, and oral administration in man. J Clin Endocrinol Metab 78: 693-698.
10. Okano M, Nishitani Y, Sato M, Ayaka I (2010) Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects. Drug Test Anal 2: 548-556.
11. Graul AI, Prous JR (2006) The Year's New Drugs: A Historical and Research Perspective on the 41 New Products that Reached their First Markets in 2005. Drug News & Perspectives (Prous Science) 19: 33.
12. Cox HD, Hughes CM, Eichner D (2015) Detection of GHRP-2 and GHRP-6 in urine samples from athletes. Drug Test Anal 7: 439-444.
13. Gaudiano MC, Valvo L, Borioni A (2014) Identification and quantification of the doping agent GHRP-2 in seized unlabelled vials by NMR and MS: a case-report. Drug Test Anal 6: 295-300.

14. Kohler M, Thomas A, Geyer H, Michael P, Wilhelm S, et al. (2010) Confiscated Black Market Products and Nutritional Supplements with Non-Approved Ingredients Analyzed in the Cologne Doping Control Laboratory 2009. Drug Test Anal 2: 533–537.

15. Thomas A, Kohler M, Mester J, Hans G, Wilhelm S, et al. (2010) Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement. Drug Test Anal 2: 144-148.

16. WADA. The 2015 Prohibited.

17. Okano M, Sato M, Ikekita A, Shinji K (2010) Determination of growth hormone secretagogue pralmorelin (GHRP-2) and its metabolite in human urine by liquid chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun. Mass Spectrom 24: 2046-2056.

18. Thomas A, Höppner S, Geyer H, Wilhem S, Michael P, et al. (2011) Determination of growth hormone releasing peptides (GHRP) and their major metabolites in human urine for doping controls by means of liquid chromatography mass spectrometry. Anal Bioanal Chem 401: 507-516.

19. Thevis M, Thomas A, Schänzer W (2011) Doping control analysis of selected peptide hormones using LC–MS(/MS). Forensic Sci Int 213: 35-41.

20. Thevis M, Thomas A, Pop V, Schanzer W (2013) Ultrahigh pressure liquid chromatography–(tandem) mass spectrometry in human sports drug testing: Possibilities and limitations. J Chromatogr A 1292: 38-50.

21. Semenistaya E, Zvereva I, Thomas A, Mario T, Grigory K, et al. (2015) Determination of growth hormone releasing peptides metabolites in human urine after nasal administration of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin. Drug Test Anal 7: 919-925.

22. Pinyot A, Nikolovski Z, Bosch J. (2010) On the use of cells or membranes for receptor binding: growth hormone secretagogues. Anal Biochem 399: 174-181.

23. Leyris JP, Roux T, Trinquet E (2011) Homogeneous time-resolved fluorescence-based assay to screen for ligands targeting the growth hormone secretagogue receptor type 1a. Anal Biochem 408: 253-262.

24. Liu Y, Shao XX, Zhang L, Ge Song, Ya LL, et al. (2015) Novel bioluminescent receptor-binding assays for peptide hormones: using ghrelin as a model. Amino Acids 47: 2237-2243.

25. Kojima M, Hosoda H, Date Y, Nakazato N, Matsua Y, et al. (1999) Ghrelin is a Growth- Hormone-Releasing Acylated Peptide from Stomach. Nature 402: 656–660.

26. Pihoker C, Kearns GL, French D, Cyris YB (1998) Pharmacokinetics and pharmacodynamics of growth hormone-releasing peptide-2: a phase I study in children. J Clin Endocrinol Metab 83: 1168-1172.

27. Cabrales A, Gil J, Fernández E, Valenzuela C, Hernandez F, et al. (2013) Pharmacokinetic study of Growth Hormone-Releasing Peptide 6 (GHRP-6) in nine male healthy volunteers. Eur J Pharm Sci 48: 40-46.

28. Ferro P, Gutiérrez-Gallego R, Bosch J, M farre (2015) Fit-for purpose radioreceptor assay for the determination of Growth Hormone Secretagogues in urine. J Biomol Screen 20: 1268-1276.

29. Pinyot A, Nikolovski Z, Bosch J, Gerard SS, Shinji K, et al. (2012) Growth hormone secretagogues: out of competition. Anal Bioanal Chem 402: 1101-1108.

30. Leuenberger N, Lamon S, Robinson N, Giraud S, Saugy M (2011) How to confirm C.E.R.A. doping in athletes' blood? Forensic Sci Int 213: 101-103.

31. Lund H, Snilsberg AH, Halvorsen TG, Peter H, Leon R (2013) Comparison of newly developed immuno-MS method with existing DELFIA (®) immunoassay for human chorionic gonadotropin determination in doping analysis. Bioanalysis 5: 623-630.

32. Holt RI, Böhning W, Guha N, Bartlett C, Cowan DA, et al. (2015) The development of decision limits for the GH-2000 detection methodology using additional insulin-like growth factor-I and amino-terminal pro-peptide of type III collagen assays. Drug Test Anal 7: 745-755.

33. Bidlingmaier M, Suhr J, Ernst A, Wu Z, Keller A, et al. (2009) High-sensitivity chemiluminescence immunoassays for detection of growth hormone doping in sports. Clin Chem 55: 445-453.

34. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wise BE, et al. (2001) A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50: 1714-1719.

35. Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier K, et al. (2001) Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 24: RC19-RC21.

36. Yoshimoto A, Mori K, Sugawara A, Mukoyama M, Yhaata K, et al. (2002) Plasma ghrelin and desacyl ghrelin concentrations in renal failure. J Am Soc Nephrol 13: 2748-2752.

37. Tvarijonaviciute A, Martínez-Subiela S, Ceron JJ (2013) Influence of different storage conditions and anticoagulants on the measurement of total and acylated ghrelin in dogs: a preliminary study. Vet Rec 172: 289.

38. Hosoda H, Doi K, Nagaya N, Okumura H, Nakagawa E, et al. (2004) Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. Clin Chem 50: 1077-1080.

39. Ferro P, Krotov G, Zvereva I, Rodchenkov G, Segura J, et al. (2016) Structure-activity relationship for peptídic growth hormone secretagogues. Drug Test Anal 9: 87-95.