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Doping control analysis of small peptide hormones: an update

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Abstract

Small peptide hormones, such as desmopressin, GHRPs, GnRHs and TB-500, represent an increasingly appealing class of performance enhancing drugs to amateur and professional athletes. In the last years, laboratories have put a big effort to implement LC-MS analysis of peptides. An ever-evolving list of substances, different analytical issues compared to “classic”, small molecules, and ethical constrains for in vivo studies can be listed among the main issues form monitoring small peptide abuse.
In this work we present some recent work from the Ghent University Doping Control Laboratory on several aspects of the problem including:
- discovery/identification of new peptidic doping agents
- development of analytical methods for analysis in urine and plasma
- metabolism studies

Introduction

The use of small peptide hormones (molecular weight < 2 kDa) as performance-enhancing or masking agents have become increasingly popular in the last years. Several classes have reached the focus of sport drug testing, including vasopressin analogues (desmopressin), growth hormone-releasing peptides (GHRPs), gonadotropin-releasing hormone (GnRH) agonists and the thymosin beta 4 fragment TB-500 [1-3]. Though having different pharmacological activities, these peptides can be grouped together because they present similar analytical issues. It is in fact currently possible to screen for all small peptides and metabolites in a single run [4].
Another major issue is represented by the fact that several of them have found their way on to black markets, particularly through online trade [3,5-7].
Furthermore, since a good part of these peptides are non approved for any therapeutic use, it is not possible to perform administration studies to investigate urinary excretion and metabolism.
Having considered these issues, DoCoLab has performed research on this class of doping agents during the last years, focusing prevalently on the following aspects:
· Identification of emerging peptidic doping drugs illegally traded in the black markets
· Development of LC-MS methods for screening and confirmatory analysis of peptide hormones in urine and plasma
· Development of *in vitro* models for the study of peptide metabolism
In this work, the most recent achievements obtained by our laboratory are briefly discussed and summarized.

Experimental

Identification of black market peptides

The content of the vials was dissolved in water at a concentration of 1-5 mg/mL. Direct injection of the solution was first performed. In some cases, trypsin digestion was performed before UHPLC-HRMS analysis. The UHPLC system consisted of an Accela LC (Thermo Scientific, Bremen, Germany) equipped with degasser, Accela 1250 pump, autosampler thermostated at 10°C and a heated column compartment. LC separation was performed using a Zorbax SB-C8, from Agilent Technologies (Böblingen, Germany). The mobile phases were 0.2% formic acid (FA) in water (H_2O) and 0.2% FA in acetonitrile (ACN).

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HRMS characterization was performed using a Q-Exactive benchtop, Orbitrap™-based mass spectrometer (Thermo Scientific, Bremen, Germany). FSMS experiments were followed by targeted MS/MS (tMSMS) experiments at a normalized collision energy (NCE) optimized for each peptide. Mass tolerance was set at 5 ppm. For molecular mass determination after spectral deconvolution, tolerance was set at ±0.5 Da. For peptides with molecular weight < 8 kDa, 100% of the sequence was covered both in top-down and bottom-up identifications via de novo sequencing. For peptides with molecular weight > 8 kDa, at least 5 tryptic peptides were characterized in their amino acid sequences.

Detection methods in plasma and urine

Sample preparation was based on previous works [1,4]. Figure 1 shows in detail the sample preparation for both plasma and urine. The same LC-HRMS system previously described for identification of black market products was used for screening analysis.

The aqueous solvent (A) consisted of 0.2% of FA in H_2O, and the organic phase (B) was ACN with 0.2% FA. The gradient started at 1% B for 5 min, rose to 35% B in 20 min, rose to 90% B in 2 minutes and was followed by riequilibration at 1% B for 8 min, with a resulting overall runtime of 35 min. The Q-Exactive benchtop quadrupole Orbitrap operated both in full scan mode from *m/z* 300 to 1500 and in targeted single ion monitoring (t-SIM) at 70,000 resolving power.

The method for confirmation in urine was developed on a TSQ Quantum Discovery triple-stage quadrupole mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive multiple reaction monitoring (MRM) mode. Chromatographic conditions were the same as described for the screening method.

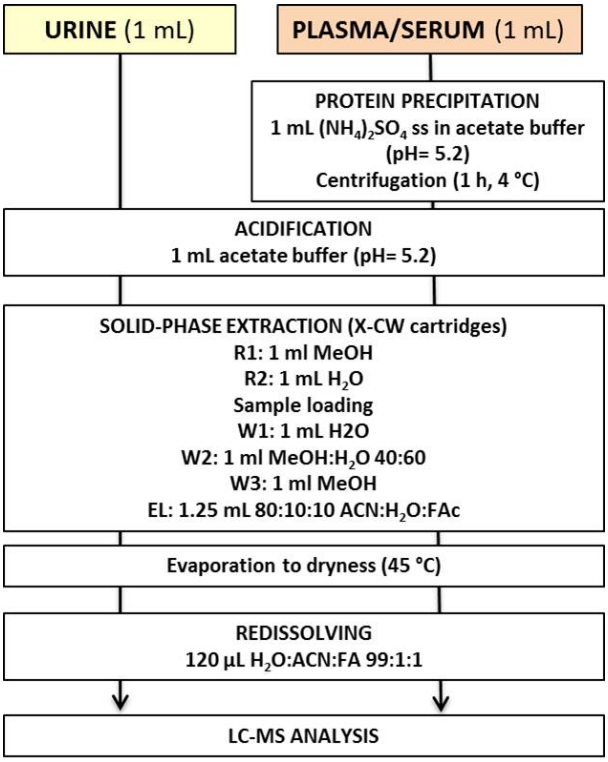

Figure 1. Schematization of sample preparation for urine and plasma.

Peptide stability

In order to verify peptide stability, 10 ng/mL aqueous solutions of peptides were incubated at 5 and 37°C. Samples were collected up to 48 hours and directly analysed.

In vitro metabolism

Alternative *in vitro* models, including human liver microsomes and S9 fraction have been investigated and compared with already described *in vitro* models. However, a manuscript dedicated to this part of the work has been prepared and is currently under review.

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Results and Discussion

Black market peptides

Identifying a new peptidic doping agent is the first step towards the development of efficient methods of detection. MS/MS experiments are performed both for *top-down* (analysis of the intact peptide) and *bottom-up* (proteolytic digestion of proteins prior to analysis by mass spectrometry of the peptide fragments). In MS/MS experiments, specific precursor ions, corresponding to the intact peptide can be selected to be fragmented. The resulting MS/MS spectra present fragment ion series which can be used to determine the amino acid sequence. Then, the sequence can be matched with available online databases. This is very useful whenever a representative of a new class of peptides is discovered (e.g.: TB-500).

However, the number of doping-related peptide is relatively small compared to the whole proteome. Therefore, an internal data base has been created, containing sequences, bruto formulas, diagnostic ions both in full scan mode and MS/MS mode, and tryptic digestion products, in order to facilitate identification of already known peptides. The database is constantly updated whenever a new peptide is identified.

In the last three years, DoCoLab, has received a total of approximatively 50-60 products from Belgian Customs. A number of peptides were identified, as listed in Table 1. Among all the peptides, CJC-1295 and GHRPs (particularly GHRP-2 and GHRP-6) were detected several times. Among all the compounds, also non-doping related peptides (melanotan II and thymopentin) were identified. Most of the vials were unlabeled, and in some cases mislabeled. In some of the vials, nothing besides excipients (mostly mannitol) was detected.

PEPTIDE	SEQUENCE
GHRP-2	(D-Ala)-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-N
GHRP-6	His-(D-Trp)-Ala-Trp-(D-Phe)-Lys-NH2
Ipamorelin	Aib-His-(D-2-Nal)-(D-Phe)-Lys-NH2
Hexarelin	His-(D-Mrp)-Ala-Trp-(D-Phe)-Lys-NH2
LHRH	Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
Leuprolide	Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt
Triptorelin	pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2
TB-500	(Acetyl)Leu-Lys-Lys-Thr-Glu-Thr-Gln
full lenght Thymosin β4	(Acetyl)MSDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES
Amidated MGF	YQPPSTNKNTKSQRRKGSTFEERK-NH2
hGH (22 kDa)	FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREETQQ KSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSPRTGQI FKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEGSCGF
sermorelin/Geref	YADAIFTNSYRKVLGQLSARKLLQDIMSRQ
CJC-1293	Y(d-A)DAIFTNSYRKVLGQLSARKLLQDIMSR-NH2
CJC-1295	Y(d-A)DAIFTQSYRKVLAQLS ARKLLQDILSR-NH2
Melanotan II	Ac-Nle-cyclo[Asp-His-D-Phe-Arg-Trp-Lys]-NH2
Thymopentin	Arg-Lys-Asp-Val-Tyr

Table 1. List of peptides identified in our laboratory in the last three years.

LC-MS detection

Though it is preferable to monitor small peptides in urine (less invasive sample collection, prolonged detection) a method for detection in plasma has been developed as well for future research purposes.

Sample preparation is a critical issue for peptide analysis. Non-specific absorption on surface, variable recovery, loss of analyte during transfer and drying steps can cause poor or irreproducible results if adequate countermeasures, such as using low-bind plasticware and limiting the number of vial transfers, are not taken. Another important factor resulted to be the pH of the sample when loaded on the column. The pH of urine can range from a low of 4.5-5 to a high of 8. pH values are also important for ion exchange interaction, since it determines whether the analyte or the resin is in the ionized or

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non-ionized form. Different buffers were therefore tested, including formiate buffer (pH 3.8), acetate buffer (pH 5.2), phosphate buffer (pH 7) and carbonate buffer (pH 9.5). Best results were obtained with acetate buffer. Concerning detection in urine, screening is performed on a UHPLC-HRMS instrument (Orbitrap Q-Exactive) in targeted SIM acquisition mode. Compared to full scan mode t-SIM yielded the best sensitivity, but full scan mode was also included in the method since it enables retrospective data evaluation for presently unknown peptides or metabolites (preventive control analysis).
Theoretical *m/z* values of the most abundant species were calculated for each peptide and then added in the inclusion list (Table 2) used in t-SIM experiments. Very abundant $[M+2H]^{++}$ pseudomolecular were generated for all the peptides except for the GHRP-2 metabolite, smaller, which showed a prevalent $[M+H]^{+}$ ion.
Limits of detection (LOD) in urine were 20 pg/mL for all the peptides, whereas in plasma it was 50-100 pg/mL. These LOD can be considered adequate for these peptides, though for most of them pharmacokinetic data are not yet available.
The use of multiplexed (MSX) option was enabled, with the possibility to acquire 10 t-SIM experiments in the same scan event. This allowed the acquisition of a higher number of data points (4-5 folds).
Confirmatory analyses are performed on a low resolution HPLC-QqQ-MS. This technique is available in all WADA-accredited laboratories, warranting global implementation. Acquisition parameters are shown in Table 3.

PEPTIDE	*m/z*
Desmopressin	535.2207
GHRP-2	409.7210
GHRP-2 metabolite (aa 1-3)	358.1761
GHRP-6	437.2296
Hexarelin	444.2374
ISTD 1 - $_{13}C$, $_{15}N$ GHRP-2 metabolite (aa 1-3)	362.1820
ISTD 2 - [deamino Cys1, Val4, D-Arg8] AVP	520.7256
LHRH	591.7914
Lys-vasopressin	528.7230
TB-500	445.2531

Table 2. Theoretical *m/z* values for peptides included in the screening method.

Peptide	Precursor Ion	Product Ions	CE (eV)
Desmopressin	535.5 (++)	328.0	16
		120.0	35
Lys-vasopressin	528 (++)	119.9	35
		225.8	33
TB-500	445.5 (++)	86.0	33
		129.0	26
LHRH	591.9 (++)	176.1	43
		249.0	26
GHRP-2	409.9 (++)	550.5	12
		276.2	18
GHRP-2 met AA 1-3	358.2 (+)	170.1	26
		269.2	11
GHRP-6	437.7 (++)	110.0	36
		324.2	22
LHRH	591.9 (++)	176.1	43
		221.0	34
Hexarelin	444.3 (++)	110.0	33
		144.1	38
ISTD 1	520.7 (++)	328.0	16
ISTD 2	362.1 (+)	269.2	11

Table 3. MRM acquisition parameters.

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Peptide stability

Knowledge of the stability of doping compounds is of utmost importance to avoid false negative findings or erroneous quantifications. As shown in Figure 2, all the peptides showed good stability in urine after 48 hours both at 5 and 37 °C.

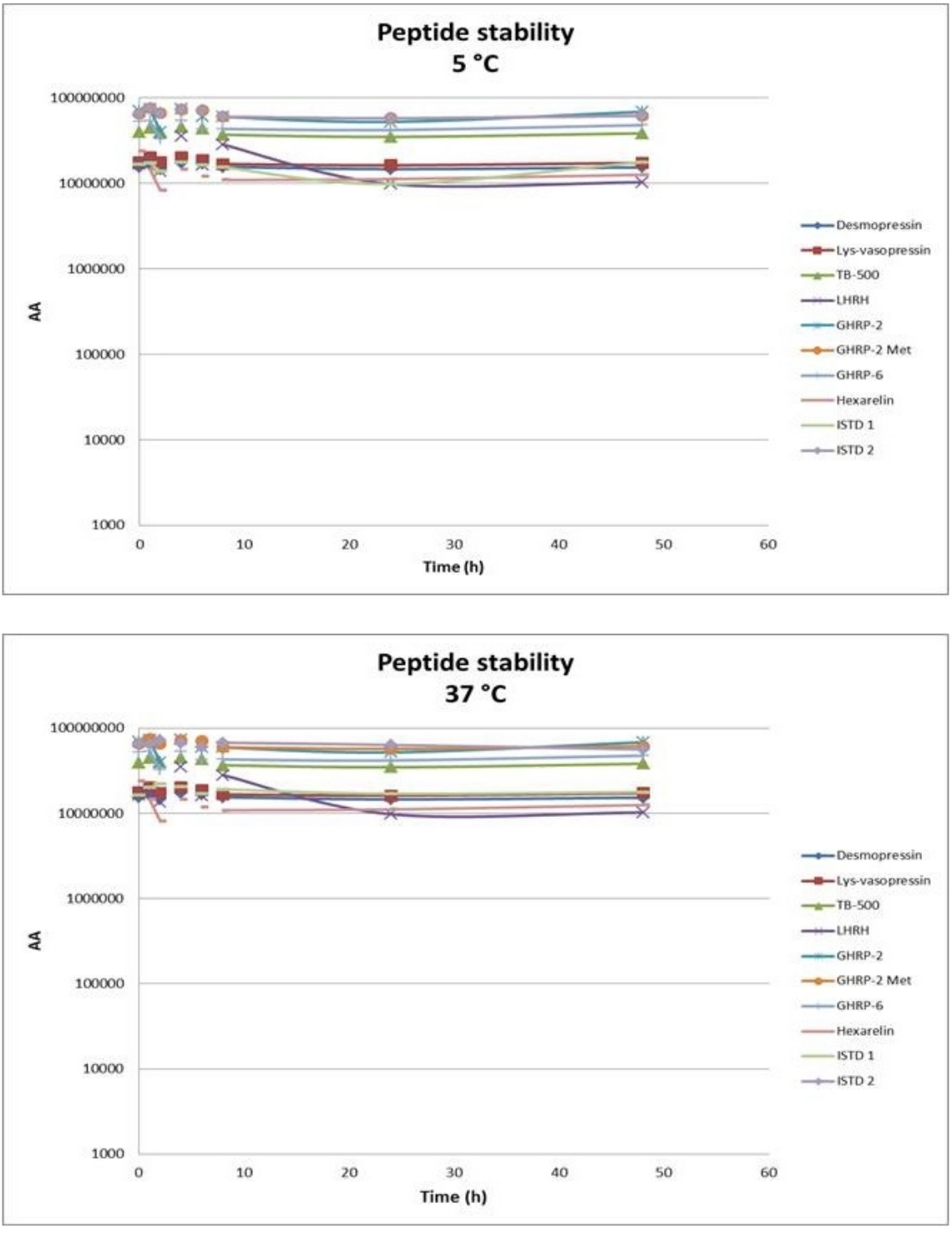

Figure 2. Peptide stability in urine at 5 and 37°C.

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Conclusions

A comprehensive approach has been used to tackle the misuse of small peptide hormones, including not only the development of suitable detection methods for screening and confirmatory analyses, but also monitoring and identifying new peptidic drugs from black markets and developing alternative models for studying metabolism of these substances. All these three aspects are important for implementing an effective testing for small peptide hormones.

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