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Development and validation of an LC-MS/MS analytical procedure for the combined detection of prohibited peptides in biological fluids

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Abstract

In the last decade a wide variety of hormone peptides were included in the WADA prohibited list. This has imposed the development of comprehensive screening procedures based on mass spectrometric techniques to meet the continuously increasing demands of rapid and specific doping control test. Here we present a method for the analysis of 14 prohibited small peptides (GHRP-1 and its metabolite, GHRP-2 and its metabolite, GHRP-4, GHRP-5, GHRP-6, LH-RH, ipamorelin, hexarelin and desmopressin and its analogues) by means of liquid chromatography/tandem mass spectrometry in selected reaction monitoring after solid phase extraction.
The procedure was validated in terms of sensitivity (LLODs ranging from 0.05 to 0.5 ng/mL), specificity (no interference were detected), recovery (> 60% with a CV % <15), matrix effect (< 35%) and reproducibility of retention times (CV% < 0.1) and of relative abundances (CV% < 15).

Introduction

Parallel to the increased number of low molecular weight substances, recently a wide variety of peptide hormones were added to the category S2" Peptide Hormones, Growth Factors, Related Substances and Mimetics" of the World Anti-Doping Agency list of prohibited substances and methods [1]. Traditionally, the analytical procedures used for the detection of macromolecules in the anti-doping field were based on the use of immunological techniques. The increasing number of macromolecules with high structural similarity with endogenous compounds has imposed the development of more selective methods based on mass spectrometry. Several methods are already published to detect peptide hormones [2-4] in biological fluids. Here we present an analytical procedure for the analysis in urine of 14 peptides.

Experimental

Chemicals and reagents

Desmopressin, vasopressin, lypressin, LH-RH, [Deamino-Cys1-Val4-D-Arg8]-Vasopressin (internal standard) and all chemicals were supplied by Sigma-Aldrich (Milano, Italy). Felypressin, GHRP-2, GHRP-6, ipamorelin and hexarelin were supplied by PepBridge (USA). GHRP-1, GHRP-4 and GHRP-5 were synthesized by Biomatik (Canada). OASIS® WCX sorbents (30 mg, 30 µm particle,1 mL) were purchase from Waters (Milano, Italy).

Analytical procedure

A urinary aliquot of 2 mL (pH 7) was fortified with 5 ng/mL of ISTD ([Deamino-Cys1-Val4-D-Arg8]-Vasopressin). The samples were loaded onto the OASIS® WCX cartridge, previously conditioned with 1 mL of methanol and 1 mL of distilled water. The samples were washed with 1 mL of water and 1 mL of methanol. Subsequently, the target analytes were eluted with 1 mL of methanol containing 10% of formic acid and with 1 mL methanol containing 25% of ammonia into a polypropylene tube. The solvent was evaporated in a vacuum centrifuge at moderate temperature (about 40 °C). The dry residue was reconstituted in 50 µL of mobile phase and injected into the LC-MS/MS system.
The chromatographic separation was performed using an Agilent 1200 Rapid Resolution Series HPLC pump (Agilent Technologies Spa, Milano, Italy), an Ascentis® C18 (50 X 2.1 mm, 2.7 µm) column from Supelco (Milano, Italy) and water and

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acetonitrile as mobile phase, both containing 0.1% of formic acid. The gradient program starts at 5% of acetonitrile and increases after 14 minutes to 85% of acetonitrile and after 1 min to 100% of acetonitrile. The column was flushed for 2 min at 100% of acetonitrile and finally re-equilibrated at 5% of acetonitrile for 4 min. The flow rate was set at 300 mL/min.
The mass spectrometer was an API4000 triple-quadrupole system (Applera, Monza, Italy) with positive electrospray ionization. The mass parameters and the selected ion transitions are reported in Table 1.

Peptide	Charge state selected	Rt (min)	Q1 (m/z)	Q3 (m/z)	Collision Offset (V)
Vasopressin	2+	5.2	543	120, 328, 757	35, 30, 27
Desmopressin	1+, 2+	6.6	535, 1069	120, 328, 1069	35, 30, 27
Felypressin	2+	5.8	521	120, 226, 358,	35,30, 27
Lypressin	2+	5.1	528	120, 129, 226	35,30, 27
GHPR-1	2+	6.9	478	209, 335, 406,	30, 25, 25
GHRP-1 metablite	2+	7.7	442	335; 406	30; 22
GHPR-2	2+	7.7	410	170, 241, 550	30, 25, 25
GHRP-2 metablite	2+	7.7	358	198, 241, 289	30, 25, 25
GHPR-4	1+	8.2	608	159, 258, 351, 444	35, 30, 30, 25
GHPR-5	1+	8.7	771	258, 350, 421, 607	45, 45, 40, 30
GHPR-6	2+	6.4	437	159, 324, 395, 581	35, 35, 30, 30
Hexarelin	2+	6.1	444	338, 409, 595	30, 27, 27
Ipamorelin	1+ 2+	5.5	357	129, 166, 223, 335	35, 30, 25, 25
LH-RH	2+	6.2	592	221, 249	30, 27
ISTD [Deamino-Cys1-Val4-D-Arg8]-Vasopressin	2+	7.3	520	328	30

Table 1: Chromatographic and mass spectrometric conditions.

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Results and Discussion

Instrumental parameters in ESI-MS and ESI-MS/MS were optimized by infusing the standards of each analyte at a concentration of 5 µg/mL. The MS spectra were dominated by singly (for ipamorelin, desmopressin, GHRP-4 and GHRP-5) and/or doubly charged precursor ions. Product ion experiments were then performed at different collision offset voltages (30, 40, 50 and 60 V) in order to obtain a sufficient number of diagnostic fragments. At least two diagnostic fragments were identified for each compound, which is considered sufficient for an initial testing procedure that, in case of suspicious results, leads to dedicated confirmation analysis (Table 1).
The chromatographic method was optimized to obtain a satisfactory chromatographic resolution between the peptides and the biological background. Optimal results were obtained using column based on the Fused-Core® technology, a column temperature of 30 °C and formic acid as mobile phase modifier (Figure 1).

Peptide	Limits of Detection (ng/mL)	Matrix Effect (%)	CV% Relative Abundances	Recovery (%)
Vasopressin	0.10	25	10	76
Desmopressin	0.05	22	12	87
Lypressin	0.50	25	10	65
Felypressin	0.10	20	14	72
GHPR-1	0.50	22	14	71
GHPR-2	0.20	20	14	85
GHPR-4	0.20	30	13	62
GHPR-5	0.20	26	11	61
GHPR-6	0.50	32	11	66
Hexarelin	0.50	33	14	63
Ipamorelin	0.20	25	13	78
LH-RH	0.20	30	10	75

Table 2: Method validation results.

Concerning the sample preparation, the 14 analytes under investigation were efficiently extracted (recovery > 60%) with a satisfactory repeatability (CV % < 15) from urine samples using a mixed-mode weak cation exchange (WCX) sorbent and two elution steps (methanol with 25% ammonia and methanol with 10% formic acid) after two washing steps with double distilled water and methanol (Figure 2).

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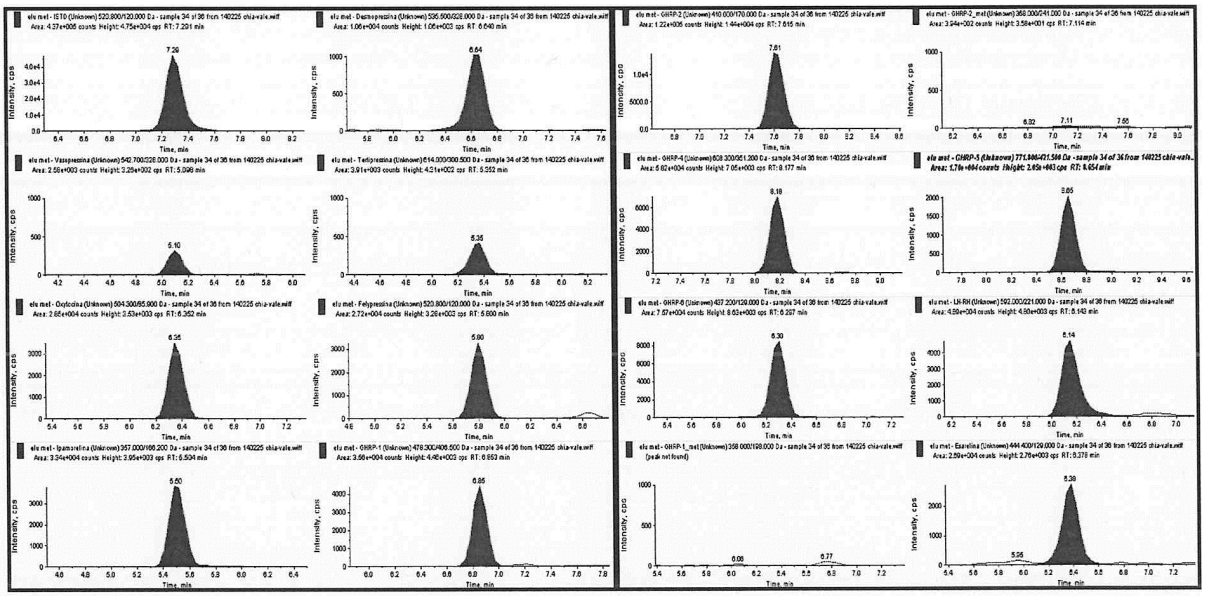

Figure 1: Urine spiked with the compounds under investigation at a concentration of 0.2 ng/mL a part from hexarelin that is present at a concentration of 0.5 ng/mL. The urine was pre-treated and analyzed using the analytical procedure reported in the experimental part.

The optimized procedure was validated according to ISO 17025 and WADA requirements. The method is sensitive (LLOD in the range of 0.05-0.5 ng/mL) specific (no interference were detected at the retention times of the analytes under investigation) and efficient (recovery > 60%) (Tab. 1). Good repeatability of retention times (CV% < 0.1) and of relative abundances (CV% < 15) was obtained (Tab. 2). Fitness of the method for doping analytical purpose was evaluated by analyzing an excretion study urine (pooled post administation fractions between 4.5h and 20.5h) collected after intravenous injection of 0.1 mg of GHRP-2 (pralmorelin dihydrochloride). Both GHRP-2 and its metabolite were detected.

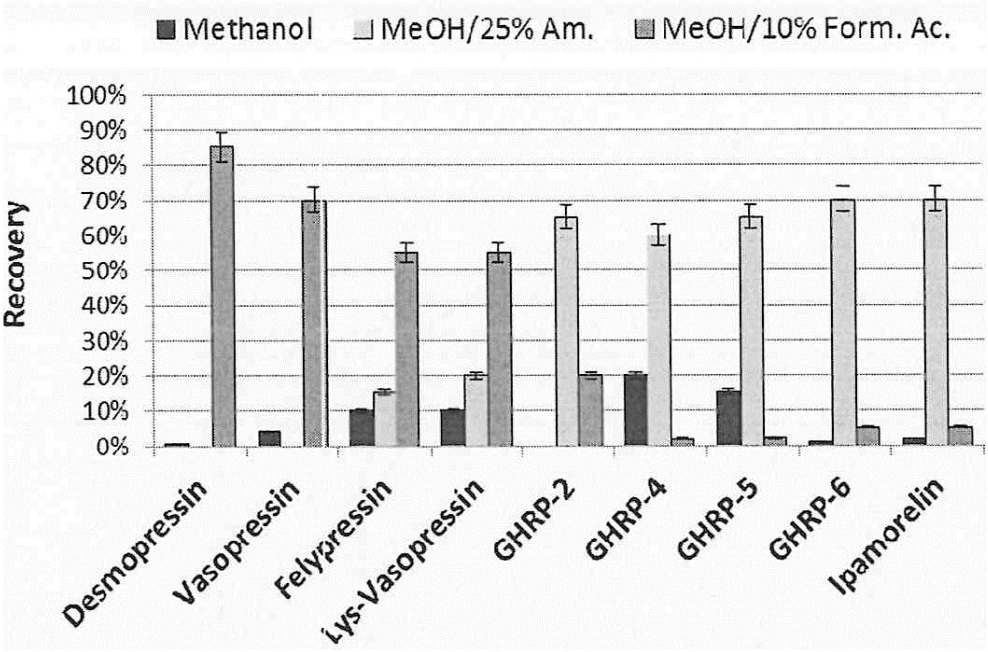

Figure 2: Percentage of recovery of 9 of the peptides under investigation.

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Conclusions

The data obtained demonstrate the capability and suitability of the LC-ESI-MS/MS analysis for the screening and confirmation of small peptides in urine samples. The analytical procedure was fully validated, evaluated on real samples and showed comparable analytical performances with respect to LC-ESI-MS procedures reported in literature. The proposed analytical procedure can be efficiently applied also to blood samples, after precipitation of proteins with acetonitrile containing 0.1% of formic acid.
In future we plan to improve the sensitivity of the present method by decreasing the column sizes (i.e. internal diameter and particle size) and by using more sensitive instrumentation. Furthermore, other prohibited peptides (i.e TB500 and ADO9604) will be introduced in the proposed analytical procedure.

References

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