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Determination of doping peptides via solid-phase microelution and accurate-mass quadrupole time-of-flight LC–MS

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ABSTRACT

A complete analytical protocol for the determination of 25 doping-related peptidic drugs and 3 metabolites in urine was developed by means of accurate-mass quadrupole time-of-flight (Q-TOF) LC–MS analysis following solid-phase extraction (SPE) on microplates and conventional SPE pre-treatment for initial testing and confirmation, respectively. These substances included growth hormone releasing factors, gonadotropin releasing factors and anti-diuretic hormones, with molecular weights ranging from 540 to 1320 Da. Optimal experimental conditions were stablished after investigation of different parameters concerning sample preparation and instrumental analysis. Weak cation exchange SPE followed by C18 HPLC chromatography and accurate mass detection provided the required sensitivity and selectivity for all the target peptides under study. 2 mg SPE on 96-well microplates can be used in combination with full scan MS detection for the initial testing, thus providing a fast, cost-effective and high-throughput protocol for the processing of a large batch of samples simultaneously. On the other hand, extraction on 30 mg SPE cartridges and subsequent target MS/MS determination was the protocol of choice for confirmatory purposes. The methodology was validated in terms of selectivity, recovery, matrix effect, precision, sensitivity (limit of detection, LOD), cross contamination, carryover, robustness and stability. Recoveries ranged from 6 to 70% (microplates) and 17–95% (cartridges), with LODs from 0.1 to 1 ng/mL. The suitability of the method was assessed by analyzing different spiked or excreted urines containing some of the target substances.

1. Introduction

New recombinant small peptidic therapeutics have emerged in the doping field during the last decade, as proven by the analysis of samples confiscated by customs or seized in operations against the doping drug trade conducted by security bodies in different countries [1–4]. These species are mainly included in the 2017 World Anti-Doping Agency (WADA) list of prohibited substances in the categories S0 (non-approved substances), S2 (peptide hormones, growth factors, related substances and mimetics) and S5 (diuretics and masking agents) [5]. Among them, growth hormone releasing peptides and secretagogues (GHRPs and GHSs, respectively) have the ability to increase natural growth hormone (hGH) plasma levels and potentially mask illicit use of recombinant growth hormone (rGH) by elevating the derived suppressed levels of hGH, thus providing clear performance enhancement [6–8]. Gonadotropin releasing hormone (GnRH) and its analogs are claimed to raise endogenous luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone levels, among other potential benefits for doping purposes [9,10]. Anti-diuretic small hormones such as desmopressin can be misused in sports environments due to their masking properties related to their impact on the athlete's biological passport (ABP) hematic parameters [11].

Accredited anti-doping laboratories must henceforth control the presence of these newly developed peptidic-based substances and/or their metabolites in routine analysis. Since these species are not structurally related to the analytes routinely determined in sports drug testing, new sample preparation procedures and instrumental analysis methods specifically developed and validated for small peptides determination are required [12–14]. Weak cation exchange (WCX) solid-phase extraction (SPE) or immunoaffinity purification and analysis by LC–MS (triple quadrupole or accurate mass Orbitrap) in urine or plasma samples have usually been the techniques of choice for the screening of these kinds of compounds [15–21]. More recently, a method employing ion-mobility time-of-flight MS after direct urine injection has been developed [22]. Overall, the protocols published until date imply both tedious and time consuming SPE pretreatment steps, including a

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Table 1
Amino acid sequence, molecular formula and monoisotopic mass of target compounds.

Substance	Amino acid sequence	Molecular formula	Monoisotopic mass
Desmopressin	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-(D-Arg)-Gly-NH_2 (disulfide bond Mpa-Cys).	$C_{46}H_{64}N_{14}O_{12}S_2$	1068.4270
Lypressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2 (disulfide bond Cys-Cys)	$C_{46}H_{65}N_{13}O_{12}S_2$	1055.4317
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (disulfide bond Cys-Cys)	$C_{46}H_{65}N_{15}O_{12}S_2$	1083.4379
Felypressin	Cys-Phe-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2 (disulfide bond Cys-Cys)	$C_{46}H_{65}N_{13}O_{11}S_2$	1039.4368
Terlipressin	Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2 (disulfide bond Cys-Cys)	$C_{52}H_{74}N_{16}O_{15}S_2$	1226.4961
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	$C_{55}H_{75}N_{17}O_{13}$	1181.5730
Leuprolide	pGlu-His-Trp-Ser-Tyr-(D-Leu)-Leu-Arg-Pro-NHC_2H_5	$C_{59}H_{84}N_{16}O_{12}$	1208.6455
Buserelin	pGlu-His-Trp-Ser-Tyr-(D-$(CH_3)_3$C-Ser)-Leu-Arg-Pro-NHC_2H_5	$C_{60}H_{86}N_{16}O_{13}$	1238.6560
Triptorelin	pGlu-His-Trp-Ser-Tyr-(D-Trp)-Leu-Arg-Pro-Gly-NH_2	$C_{64}H_{82}N_{18}O_{13}$	1310.6309
Deslorelin	pGlu-His-Trp-Ser-Tyr-(D-Trp)-Leu-Arg-Pro-NHEt	$C_{64}H_{83}N_{17}O_{12}$	1281.6407
Fertirelin	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHEt	$C_{55}H_{76}N_{16}O_{12}$	1152.5829
Goserelin	pGlu-His-Trp-Ser-Tyr-(D-^{t}Bu-Ser)-Leu-Arg-Pro-(Aza-Gly)-NH_2	$C_{59}H_{84}N_{18}O_{14}$	1268.6414
Nafarelin	pGlu-His-Trp-Ser-Tyr-(D-β-Nal)-Leu-Arg-Pro-Gly-NH_2	$C_{66}H_{83}N_{17}O_{13}$	1321.6356
Peforelin	pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2	$C_{59}H_{74}N_{18}O_{14}$	1258.5632
GHRP-1	Ala-His-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-NH_2	$C_{51}H_{62}N_{12}O_7$	954.4864
GHRP-2	(D-Ala)-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys-NH_2	$C_{45}H_{55}N_9O_6$	817.4276
GHRP-2 deamidated (GHRP-2 M1)	(D-Ala)-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys	$C_{45}H_{54}N_8O_7$	818.4116
GHRP-2 [AA 1–3] (GHRP-2 M2)	(D-Ala)-(D-β-Nal)-Ala	$C_{19}H_{23}N_3O_4$	357.1689
GHRP-3	(Aib)-(D-Trp)-(D-Pro)-(D-Ile)-Arg-NH_2	$C_{32}H_{50}N_{10}O_5$	654.3966
GHRP-4	(D-Trp)-Ala-Trp-(D-Phe)-NH_2	$C_{34}H_{37}N_7O_4$	607.2907
GHRP-4 deamidated (GHRP-4 M1)	(D-Trp)-Ala-Trp-(D-Phe)	$C_{34}H_{36}N_6O_5$	608.2747
GHRP-5	Tyr-(D-Trp)-Ala-(D-Trp)-Phe-NH_2	$C_{43}H_{46}N_8O_6$	770.3540
GHRP-6	His-(D-Trp)-Ala-Trp-(D-Phe)-Lys-NH_2	$C_{46}H_{56}N_{12}O_6$	872.4446
Alexamorelin	Ala-His-(D-Mrp)-Ala-Trp-(D-Phe)-Lys-NH_2	$C_{50}H_{63}N_{13}O_7$	957.4973
Hexarelin	His-(D-Mrp)-Ala-Trp-(D-Phe)-Lys-NH_2	$C_{47}H_{58}N_{12}O_6$	886.4602
Anamorelin	Non-peptidic composition: 3-{(2R)-3-{(3R)-3-Benzyl-3-[(trimethylhydrazino)carbonyl]-1-piperidinyl}-2-[(2-methylalanyl)amino]-3-oxopropyl}-1H-indole	$C_{45}H_{55}N_9O_6$	546.3318
Ipamorelin	Aib-His-(D-β-Nal)-(D-Phe)-Lys-NH_2	$C_{38}H_{49}N_9O_5$	711.3857
Ibutamoren	Non-peptidic composition: 2-Amino-2-methyl-*N*-[1-(1-methylsulfonylspiro[2H-indole-3,4′-piperidine]-1′-yl)-1-oxo-3-(phenylmethoxy)propan-2-yl]-propanamide	$C_{27}H_{36}N_4O_5S$	528.2406

Mpa = mercaptopropionic acid; pGlu = pyroglutamic acid; D-$(CH_3)_3$C-Ser = *O*-(*tert*-butyl)-D-seryl; Aza-Gly = H_2N-NH-COOH; Nal = naphtylalanine; D-Mrp = 2-methyl-D-tryptophane; Aib = aminoisobutyric acid.

significant time lag for sample drying before the final reconstitution step.

Pursuing our interest in the search of fast, high-throughput, and environmentally friendlier protocols by means of automation and/or miniaturization, which could be easily implemented in any doping control laboratory [23,24], here we present a complete analytical assay for the determination of 28 doping-related substances in urine through Q-TOF HPLC–MS analysis, following a microelution-based SPE purification (initial testing), or conventional SPE pre-treatment (confirmation). The combination of SPE on microplates with the high resolution and accurate mass capabilities of a Q-TOF system results in a simple, straightforward and cost-effective solution for the determination of small peptidic recombinant drugs in urine samples. Target analytes include growth hormone releasing factors (GHRP-1, -2, -3, -4, -5, -6, alexamorelin, hexarelin, anamorelin, ipamorelin and ibutamoren), gonadotropin releasing factors (LHRH, leuprolide, buserelin, triptorelin, deslorelin, fertirelin, goserelin, nafarelin and peforelin) and anti-diuretic hormones (desmopressin, lypressin, vasopressin, felypressin and terlipressin). Three metabolites (GHRP-2 deamidated (GHRP-2 M1), GHRP-4 deamidated (GHRP-4 M1) and GHRP-2 [AA 1–3] (GHRP-2 M2)) were also included in this work. GHRP-2 [AA 1–3] is considered the main metabolite to mark GHRP-2 consumption [25,26]. On the other hand, C-terminus deamidation of GHRPs due to amidase activity has been observed in considerable extent after in vivo/in vitro experiments conducted by several authors [27,28]. The validated procedures fulfilled the concentration levels established by WADA for these kinds of compounds [29].

Full data about both sample preparation and instrumental analysis for both initial and confirmatory methods, evaluation of the optimal experimental conditions, validation results and applicability of the developed methodology to real samples are shown throughout this work.

2. Material and methods

2.1. Chemicals and reagents

All solvents and reagents used were of analytical grade. Acetonitrile and methanol were from Merck (Darmstadt, Germany). Formic acid was from Scharlau (Sentmenat, Spain). Sodium hydroxide, hydrochloric acid fuming (37%), potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) were from Merck (Darmstadt, Germany). Ultrapurified water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

A stable isotope-labelled peptide was used as a representative internal standard (ISTD) for the 28 substances included in this work: GHRP-2 M1 ((D-Ala)-(D-β-Nal)-Ala-Trp-(D-Phe)-Lys) labelled in lysine (6 ^{13}C and 2 ^{15}N, β-Nal = β-naphthylalanine) was synthesized by the Proteomics Unit, Spanish National Biotechnology Centre (CSIC, Madrid, Spain), with a purity > 95%.

Ipamorelin, GHRP-1, GHRP-2, GHRP-4, GHRP-5, hexarelin, LHRH (gonadorelin) and triptorelin were purchased from Abbiotec, LLC (San Diego, CA, USA); GHRP-6, leuprolide, buserelin, deslorelin, fertirelin, goserelin, nafarelin, peforelin and terlipressin were obtained from Bachem Ltd. (Bubendorf, Switzerland); desmopressin, lypressin and vasopressin were purchased from Sigma (Deisendorf, Germany); anamorelin and ibutamoren were obtained from Toronto Research Chemicals (Toronto, Canada); felypressin was purchased from European Pharmacopoeia (Strasbourg, France); alexamorelin, GHRP-3, GHRP-2 M1, GHRP-2 M2 and GHRP-4 M1 were synthesized by the Proteomics Unit, Spanish National Biotechnology Centre (CSIC, Madrid, Spain), with purities > 95% for all of them.