Recent advances in peptide analysis by LC-MS for doping controls

Abstract Doping with peptide-based drugs has gained more and more relevancy since the last decades. Thus, analytical methods are required to enable efficient sports drug testing for this class of prohibited substances. One of the most important tools for peptide analysis is the combination of liquid chromatography coupled to mass spectrometry (LC-MS). Within this manuscript recent advances and developments in the analysis of peptide hormones by means of LC-MS are described with a special focus on the analysis of peptides and proteins according to the list prohibited substances in professional sport (*e.g.* insulins, growth hormone releasing peptides, IGF-I (and analogs), somatotropin etc.).

Keywords **Mass spectrometry, peptide hormones.**

Résumé **Les avancées récentes de l'analyse peptidique par LC-MS**

Le dopage au moyen de médicaments à base de peptides est devenu de plus en plus pertinent au cours des dernières décennies. Par conséquent, des tests de dépistage efficaces de telles substances interdites dans le sport sont devenus nécessaires. L'un des outils les plus importants pour l'analyse des peptides est la chromatographie en phase liquide couplée à la spectrométrie de masse (LC-MS). On décrit ici quelles sont les avancées récentes dans l'analyse LC-MS des hormones peptidiques, en insistant particulièrement sur l'analyse des peptides et des protéines incluses dans la liste des substances interdites dans le sport professionnel (par exemple, les insulines, les peptides libérant l'hormone de croissance, l'IGF-I (et les analogues), la somatotropine, etc.).

Mots-clés **Spectrométrie de masse, hormones peptidiques.**

A large number of different substances is banned in sport that are considered as doping agents. Amongst these, peptide hormones play an important role and they are found on the World Anti-Doping Agency (WADA) Prohibited List in different categories, most prominently though under S2 (Peptide hormones, growth factors, related substances, and mimetics) [1]. The detection of these substances represents a major challenge in doping analysis, as they differ greatly in their structural properties from classic doping substances (such as steroids, stimulants, narcotics, etc.).

Mass spectrometry of prohibited peptide hormones

In addition to established analytical methods based on ligand-binding assays (LBA), which are used to analyze peptide hormones such as human growth hormone, erythropoietin, etc., more and more LC-MS-based methods have been developed in recent years [2] .In particular, the availability of high-resolution mass spectrometers coupled to liquid chromatographs, which are available in most doping control laboratories, enables effective analysis. Examples of such methods are implemented for insulins, growth hormone releasing peptides, gonadorelin (and analogs), IGF-I (and analogs), corticorelin, mechano growth factors and others [3-6]. These methods are all based on the measurement of intact peptides (top-down), but analysis after enzymatic hydrolysis (bottom-up) has also been realized in many instances (*e.g.* myostatin inhibitors, activin receptor activators, chorionic gonadotrophin etc.) [7]. The main factor for analyzing the hormone intact or after hydrolysis is the size or molecular mass of the peptide/protein. The *figure 1* shows the distribution of existing analytical approaches (with examples) using top-down respectively bottom-up analysis according to the molecular mass (ranging from 1-150 kDa).

Lower molecular mass peptides (up to 2 kDa) are commonly analyzed after simple solid-phase extraction procedures or even by direct urine injection, and modern mass spectrometers enable low detection limits in the sub-ng/mL level [8]. With increasing molecular masses (2-12 kDa), the sample preparation procedures become more and more complex with assays using immune-purification or mixed-mode solid-phase extraction. A recent development here is that the sample preparation procedures change from laborious and time/cost-intensive immunoaffinity purification to much more simplified solid-phase extraction procedures [5]. This is mainly enabled by the utilization of modern high resolution mass spectrometer

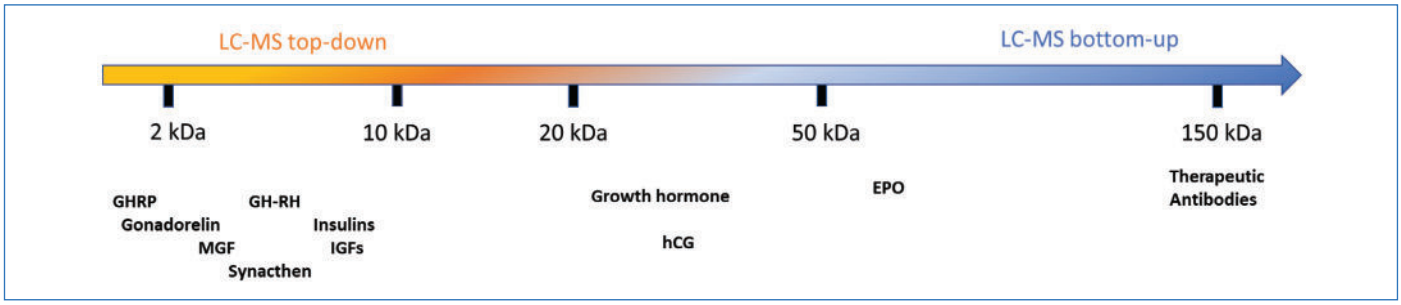

Figure 1 - Distribution top-down - resp. bottom-up LC-MS-based assays for peptide hormones according to their molecular masses. (GHRP: growth hormone releasing peptide; MGF: mechano growth factor; GH-RH: Growth hormone releasing hormone; IGF: insulin like growth factor, hCG: human chorionic gonadotrophin; EPO: erythropoietin).

with a resolution power of > 100 000 full width at half maximum (FWHM) in combination with adapted liquid chromatographic conditions allowing the analysis of less purified sample extracts. These generic sample preparation procedures further allow for the simultaneous analysis of different classes of peptide hormones with a molecular mass between 2 and 10 kDa.

While the analysis of synthetic peptides, which were not produced in the human organism, is covered by simple qualitative analysis, the monitoring of endogenous peptide hormone levels requires reliable quantitative analysis. Therefore, the quantitative determination of several peptide hormones by means of LC-MS has received growing interest in sports drug testing. Target endogenous peptides are IGF-I, P-III-NP and hCG [3, 9, 10]. The reliable quantification is facilitated by the utilization of stable isotope-labelled (SIL) internal standards, which are available for many peptide hormones nowadays. These SIL standards are added to the sample aliquot at the beginning of the sample preparation procedure and ideally compensate for analyte losses or confounding effects at any sample preparation and analysis step. While for the ideal SIL peptide analogues all amino acids are labelled (*e.g.* ^{15}N), also peptides featuring single amino acid labelling only are available and enable the desired control of the processes.

Challenges and regulations in doping control peptide analysis

Due to its unrivaled specificity, mass spectrometric analysis of peptide hormones represents the state-of-the-art methodology in doping controls. Several potentially performance-enhancing peptide hormones are included in the list of prohibited substances issued by WADA. Under consideration of the molecular mass of the peptide/protein, the analysis is realized by top-down- respectively bottom-up analysis. Major challenges result from the fast (and partly) unknown metabolism of peptides after parenteral administration and the, consequently, prevailing low concentrations in blood and urine. Generally, the analysis of blood (plasma or serum) samples offers the better conditions due to the commonly higher concentrations and the circulation of the intact hormone. Renal clearance is not entirely investigated for some peptide hormones yet.

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