"New challenges in insulin detection; liposome-vehiculated insulins"

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Project Overview

Liposome-vehiculated insulins (LVIs) are a class of insulins not yet officially marketed that should allow the intake of insulins by non-invasive routes (i.e. orally and by inhalation). This class of products, at present officially available only as prototypes, have been designed to optimize the pharmacokinetic properties of recombinant insulins produced for therapeutic uses, with the aim of developing insulin-based pharmaceutical preparations to be administered also by non-invasive routes (i.e. orally or by inhalation), but could in principle be illicitly abused also as doping agents in sport. As such, LVIs fall into two classes of the 2013 WADA Prohibited List International Standard, that are class "S0" ("Non approved substances") and class "S2" ("Peptide Hormones, Growth Factors and Related Substances"). We therefore plan to develop a specifically designed analytical strategy to detect the administration of LVIs by the analysis of biological fluids. We expect that two parallel analytical procedures may be necessary, and specifically (i) a flow cytofluorimetric method capable of identifying the presence of intact or poorly biotransformed LVIs in blood matrices (whole blood, plasma, serum) and (ii) a LC-MS/MS based method for the profiling of the breakdown products (mainly phospholipids and sphingomyelins) of liposomes, excreted in urine. In addition to this, we also plan to investigate whether liposomes themselves, either as components of LVIs preparations and/or as "free" drug delivery systems, can interfere with the procedures currently followed within the network of WADA accredited laboratories for the detection of the intake of non endogenous insulins (and as such falling into the class "M2. Chemical and Physical Manipulation" of the WADA prohibited list).

Results and Conclusions:

The detection, and ideally quantification, of doping agents that still remain "invisible" to the anti-doping laboratories are one of the most urgent challenges for the anti-doping community. To be "invisible", a doping agent needs to satisfy one or more of the following conditions: (i) to be still unknown; (ii) to be identical to an endogenous substance, (iii) to be present in the biological fluids in a concentration smaller than the limit of detection of the available analytical methods, and/or (iv) to be masked by masking agents that are themselves unknown. This research project has specifically addressed this last point.

In the last few years, novel and potentially more effective masking agents have been considered in sport doping. Among them, drug delivery systems (DDS) seem to be particularly attractive to cheaters. DDS may indeed be used to alter the absorption, release, distribution and excretion profile of

prohibited drugs, making their detection by the WADA-accredited laboratories more problematic.

The starting point of the study is based on the hypothesis – confirmed by the evidence gathered in the framework of anti-doping investigations carried out by the legal Authorities in our Country – that athletes may misuse a peculiar class of DDS, and specifically those constituted by phospholipidic liposomes, to obtain a "masking effect" when administered in association with, or consecutively to, prohibited substances, making the detectability of the latter more problematic for the anti-doping laboratories.

Consequently, the main objective of this project has been the development of effective, sensitive and effective analytical procedures allowing the detection of the intake of liposome-based DDS from the analysis of blood and/or urine samples. The main results we have obtained can be summarized as follows:

I. the size and morphology of liposomes make possible their detection by analytical techniques originally developed for the analysis of cells and other blood components; specifically, the dimensions of liposomes are in a range that can successfully be detected by flow cytofluorimetric techniques, and especially by automated hematological analyzers capable of monitoring the dimensional region of the liposomes (in our case, an ADVIA 120 analyzer monitoring the events recorded in the "blasts" and "noise" areas of the cytogram;
II. the different classes of phospholipids, that are the basic constituents of liposomes, can successfully be identified, both in pharmaceutical-grade products and in biological fluids, by liquid chromatographic-mass spectrometric techniques; in details, the newly developed methods allow the selective identification of the main classes of phospholipids, based on a combination of their chromatographic profile and of their mass spectrometric pattern, using different polarities for the electrospray ionization;
III. the intake of pharmaceutical-grade liposomes (and specifically, of the product "Liposom Forte®" that is commercialized in our Country) can be detected by the above mentioned LC-MS methods (specifically, UHPLC-HRMS in full scan acquisition), with a window of detection that, although relatively short (< 24 hours from the administration) can in principle be applied for the analysis of biological samples routinely processed by the WADA accredited anti-doping laboratories;
IV. the mass spectrometric pattern of the different classes of phospholipids can also be used for the development of targeted UHPLC-MS/MS methods, in more selective acquisition modes (e.g. SRM), following the preliminary characterization of any liposome formulations, whenever available, thus extending the window of detection of their characteristic breakdown products.

The above results may also constitute a valid starting point to assess the actual diffusion of the use of liposome-based drug delivery systems among

the athletes, in the case the WADA will plan to include this class of substances among those covered by the “monitoring program.