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How we risk: liposomes and steroids

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The fight against the illicit use of doping substances and methods to produce an unfair advantage in sport is a race against time. A substance or a method shall be included in the Prohibited List also in the case there is medical or other scientific evidence that the substance or method has the potential to mask the use of other prohibited substances or prohibited methods [1]. In this work, which follows a preliminary study whose results have already been presented [2] the potential masking effect of liposomes on the detection of androgenic anabolic steroids (AAS) has been investigated. Steroids were chosen as drugs model since they still represent the most abused class of performance-enhancing drugs.

Liposomes are artificial vesicles composed of lipid bilayers. They typically consist of phospholipids such as phosphatidylcholine and phosphatidylglycerol and cholesterol, a waxy steroid. Since liposomes were first described, in 1961, the attention of scientists in different areas of research focused on their ability to encapsulate large amounts of both small molecules and proteins. They were also studied as models of cell membranes[3].

In pharmaceutics, nano-sized liposomes are mainly utilized as drug delivery systems (DDSs). Compounds which in a classical route of administration could lead to severe toxicity (like intravenous solution of antitumor drugs) often showed a relevant improvement in their therapeutic index when they are administered as liposomal formulations[4].In this case, the pharmacokinetics (PK) of the carried drug becomes secondary to that of the carrier. Since liposomes can potentially circulate in the blood in a stable form and do not undergo glomerular filtration, the drug would be released slowly and for longer time. This leads to a decrease of metabolic deactivation and renal excretion, and allows for the use of lower doses and less toxic treatments.

Theoretically, liposomes can mask steroid abuse in doping acting both as “body-oriented” masking agents, modulating the PK of the drugs (like diuretics do), or as “lab-oriented”, interfering indirectly with the analytical methods (like proteases do).

Several potential actions were investigated:

- potential effect of a liposome-based formulation for slow release of anabolic steroids (testosterone) on the steroid pharmacokinetic
- post-formation interaction of empty liposomes with steroids, particularly glucuronated metabolites (norandrosterone glucuronide (NAG) and noretiocholanolone glucuronide (NEG), main urinary metabolites of nandrolone
- effects on the GC-MS quantitative analysis of NAG and NEG

Finally, several analytical strategies to detect liposome-based formulations in biological fluids and/or to minimize their masking effect were considered.

Materials and methods

Chemicals and reagents

Testosterone, d3-testosterone, norandrosterone glucuronide (NAG), d3-norandrosterone, noretiocholanolone glucuronide (NEG), d4-noretiocholanolone, androsterone were purchased from Australian Government – National Measurement Institute (Pimbley, Australia)All chemicals were supplied by Carlo Erba (Milano, Italy). β-glucuronidase from *E. Coli*was purchased from Roche (Monza, Italy). Trizma™-Glybuffer (pH 7.4) was prepared by dissolving in 1 L of water 8.5 mg of tris(hydroxymethyl)aminomethane (Trizma™) and 170 mg of glycine hydrochloride, both purchased from Sigma-Aldrich. The derivatizing agent (TMSD) was a mixture of N-methyl-N- trimethylsilyl-trifluoroacetamide (MSTFA)/NH4I/Dithioerythritol (1000:2:4:v/w/w). MSTFA was supplied by Alfathech (Genova, Italy). Ammonium iodide (NH4I) and dithioerythritol (DTE) were supplied from Sigma–Aldrich (Milano, Italy).

Liposomes

Purebright® and Coatsome® freeze-dried liposomes were purchased from NOF Co., Ltd. (Tokyo, Japan). Purebright SL-220, specifically designed for hydrophobic drugs, consist of DSPE-PEG. EL-01-A have an anionic net surface charge and are composed of dipalmitoylphosphatidylcholine: cholesterol: dipalmitoylphosphatidylglycerol 30:40:30 μmol/vial, total lipid amount: 61 mg. EL-01-C are cationic and composed of dipalmitoylphosphatidylcholine: cholesterol: stearylamine 52:40:8 μmol/vial, total lipid amount: 57 mg. EL-01-N are non ionic and composed of dipalmitoylphosphatidylcholine: cholesterol: dipalmitoylphosphatidylglycerol, 54:40:6 μmol/vial, total lipid amount: 61 mg.

Dialysis experiments

The dialysis experiments were performed with a biotech cellulose ester membrane (Float-A-Lyzer®G2 by Spectra/Por®), MWCO: 3.5-5 kDa. Dialysis devices were first rinsed with water and then conditioned with Trizma™-Gly buffer.SL-220 liposomes were reconstituted with 2 mL of testosterone at a concentration of 50 µg/mL in MeOH. The solution was evaporated to dryness under nitrogen stream and redissolved with 2 mL of Trizma™-Gly buffer. Then, the dialysis membrane was loaded with the liposome containing solution and its body was threaded into the floatation ring. Finally, the membrane is left floating vertically in a beaker containing 100 ml of Trizma™-Gly buffer. The beaker was placed on a magnetic stirrer in order to accelerate testosterone diffusion and homogenize outer concentration. Contemporarily, a solution of free testosterone (no liposomes) at a concentration of 50 µg/mL in Trizma™-Gly buffer was loaded in a second membrane in the same conditions. Aliquots of 1 mL were collected from both outer solutions at every hour for 8 hours, and 1 mL of fresh Trizma™-Gly buffer was added to the solutions. Finally, all the samples collected were analyzed by GC-MS.First, 50 µL of d3-testosterone (10 µg/mL) were added to the samples as internal standard and 500 µLcarbonate/bicarbonate buffer (0.8 M, pH 9). Liquid liquid extraction (LLE) was performed with 3 mL of *n*-pentane. Samples were vortexed (5') and centrifuged (5'@ 22°C), then the organic phase, containing testosterone and the internal standard, was transferred to another tube. 100 ng of androsterone were added as internal standard for derivatization, then samples were evaporated to dryness. The dried extract was redissolved with 50 µL of TMSD solution and incubated (30'@ 78°C) for the derivatization. Finally, the final extract was transferred to a glass vial, ready for GC-MS analysis.

Effect on steroid recovery

The effect on steroid recovery was evaluated in Trizma™-Gly buffer. Two mL of buffer were spiked with NAG and NEG at 2 different concentrations (50 or 100 ng/mL). Then, different volumes of empty liposome solutions (40 and 100 µL of EL-01-A, EL-01-C, or EL-01-N) were added to the samples except the "blank" samples.500 µL of phosphate buffer (1 M, pH 7.4), 50 µL of β-glucuronidase from *E. coli* and the deuterated standards at the same concentration of NAG and NEG were added to urine and the sampleswere incubated for 1 h at 50 °C. After hydrolysis, 5 mL of *n*-pentane were used for LLE and the rest of the sample preparation before GC-MS analysis was the same as for the dialysis experiments.

Effect on steroid derivatization

The effect on steroid recovery was evaluated in urines from healthy volunteers. Samples were fortified with NAG, NEG, and liposomes at the same concentrations as discussed above for the study on steroid recovery. Then, samples were splitted in three operative lines. Two lines consisted of only LLE, respectively with TBME and *n*-pentane, after glucuronide hydrolysis. A third one included solid phase extraction with C18 cartridges (100 mg, Varian Inc., Harbour City, USA) before hydrolysis. Briefly, cartridges were conditioned first with 2 ml of MeOH and then with 2 mL of H_2O, before samples were loaded. After a washing step with 2 mL of H_2O, samples were eluted with H_2O:MeOH 50:50. The eluate was evaporated to dryness and redissolved in 2.5 mL of phosphate buffer. After hydrolysis, LLE was performed with *n*-pentane. The organic layer was transferred to another tube for each sample, then to the 3 experimental lines androsterone was added before derivatization and GC-MS analysis.

Gas chromatography-mass spectrometry

GC-MS analysis was performed on an Agilent Technologies 5890/5973A (Milano, Italy), in electron impact ionisation (70 eV), using a 17 m fused silica capillary column cross-linked methyl silicone, ID 0.20 mm, film thickness 0.11µm. The carrier gas was helium (flow rate: 1 mL min^{-1}, split ratio 1:10), and the temperature program was as follows: 180 °C (hold 4.5 min), 3 °C min^{-1} to 230 °C, 20 °C min^{-1} to 290 °C, 30 °C min^{-1} to 320 °C; the transfer line temperature was set at 280°C. Acquisition was carried out in selected ion monitoring. The diagnostic ions*m/z* 432 and 435 were monitored for testosterone and d3-testosterone, respectively. NA and NE resulting form the hydrolysis of NAG and NEG were monitored by using *m/*z 405.m/z 408 and 409 were used for d3-norandrosterone a d4-noretiocholanolone, respectively. m/z 272 and m/z 434 were used for androsterone monoTMS and bisTMS.

Data analysis

All the experiments were performed at least in duplicate. For dialysis experiment, the values of the concentration of testosterone were calculated by comparing the peak areas of the detected signals for testosterone with the one of the deuterated internal standard.

For calculation of the effect of liposome-steroid interaction on NAG and NEG recovery, ratio between the peak area of the detected signals for the steroids and their respective deuterated in samples containing liposomes was compared to the ratio in blank samples (no liposomes). For the calculation of the derivatization efficiency, the ratio between the peak area of the detected signals for the monofunctional derivatization product of androsterone

(andromonoTMS) and the bifunctional analogue (androbisTMS) were compared. Derivatization was considered complete only when andro mono/bis ratio in the sample was below 10%.

Results and discussion

Liposomes as "traditional" (body-oriented) masking agents

The detection of doping with testosterone(selected as the model steroid for this study) is still based on the measurement of the T/E value in urine by GC-MS [5]. Figure 1 depicts the *in vitro* release profiles of free and liposome-encapsulated testosterone obtained with dialysis method. It is evident that the diffusion of free testosterone follows a simple diffusion model (first order). On the other hand, the release of liposome-encapsulated testosterone is characterized by a constant rate (zero order), typical for drug delivery systems.

Controlled drug release can be conceptually considered a form of masking. In fact, the encapsulation of drugs in liposomes, in this case steroids, could cause relevant effects on steroid PK, and, consequently, on doping control analysis of these drugs. Liposomes (with the encapsulated drug) does not undergo glomerular filtration, with the result that the PK of the steroid becomes secondary to the liposome's one. This is supposed to reduce the urinary levels of the drug, to prolong its half life and to allow for the use of doses lower than those necessary for classic formulations such as tablets.

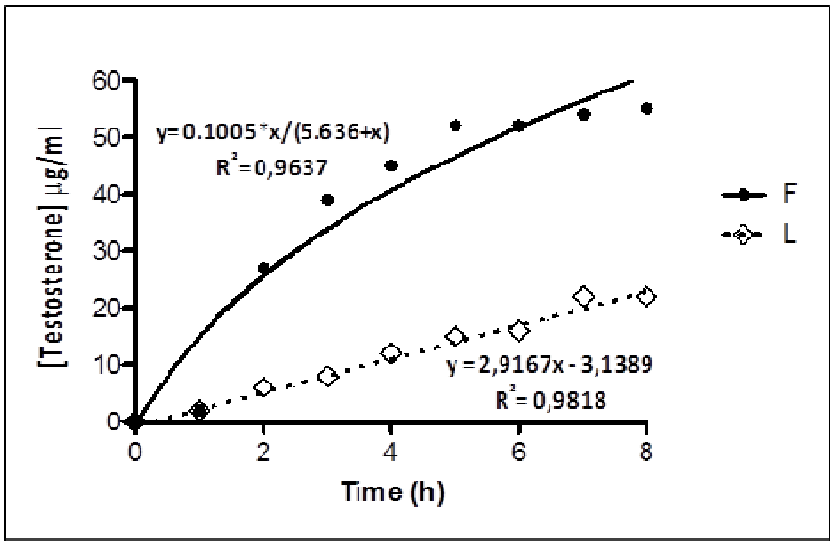

Figure 1 *In vitro* dialysis model for free (F) and liposome-encapsulated (L) testosterone.

Although the reduction of the urinary concentration is relatively small (this meaning that liposome-vehiculed synthetic AAS would still be detectable by the current methods), this effect can have dramatic consequences on the detection of doping by endogenous AAS, preliminarily based on threshold concentration values or ratios, or profiling data [6]. Even considering that an *in vitro* model for a liposomal formulation has some limitations respect to *in vivo* release (e.g.: immunogenic reactions), it is evident that liposomes can realistically alter AAS PK, prolonging their release and pharmacological effects.

Liposomes as adulterating agents (lab-oriented)

The "lab-oriented" effect can in principle be achieved by a fraudulent addition of empty liposomes to a urine sample during specimen collection. Interaction of empty liposomes with glucuronate rather than with free steroids was therefore studied, in order to evaluate the magnitude of a potential "capturing power" of steroid metabolites in the collected urine sample.Nandrolone was selected as the model AAS to investigate such effects. Nandrolone is a low abundant intermediary product synthetized during testosterone conversion to estradiol via an alternative pathway. WADA has established a threshold of 2 ng/mL for this compound, to be adjusted for the urine specific gravity [7].

The effect of the presence of empty liposomes on nandrolone analysis, particularly on nandrolone metabolites extraction and derivatisation steps was evaluated. The sample prepraration for the screening analysis of nandrolone by GC-MS is highly standardized among WADA accredited laboratories and, therefore, issues related to these two important pre-analytical steps are commonly encountered. After fortifying Trizma™-Gly buffer with nandrolone metabolites norandrosterone glucuronide and noretiocholanolone glucuronide, liposomes were added to the sample, in order to simulate an illicit addition during specimen collection. Then, ultrafiltration devices were used to retain liposomes. The steroids that were "captured" by liposomes formed complex liposome-drug that is too big to pass through the pores and therefore they were not recovered from the filtrated solution. The percentage of the steroids that were not detected in the filtrate, by comparison with a reference blank (a spiked urine with no liposomes added), corresponded to the steroids that interacted with liposomes. Results from ultrafiltration experiments shown on Figure 2, revealed that a relevant fraction (up to 60%) of the nandrolone glucuronated metabolites NAG and NEG is retained by the molecular sieves. The effect is proportional to the amount of liposomes added to the solution, as shown on Figure 3, and its intensity slightly varies with varying the nature of the liposomes.Moreover, the sum of their amount in the retained and the filtrated fraction,

expressed as standard/deuterated ratio is sometimes less than the amount in blank samples. This difference represents the percentage of NAG and NEG that results undetectable, since sample preparation procedures are not sufficient to break the interaction of the steroids with liposomes:

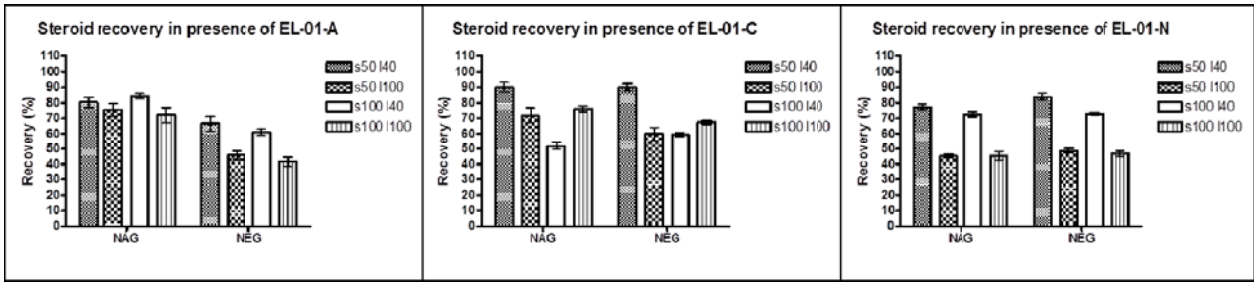

Figure 2: NAG and NEG recovery in presence of EL-01_-A, -C, and N liposomes. The experiments were performed with steroid concentration of 50 (s50 series) and 100 ng/ml (s100). 40 (l40) and 100 µl (l100) of liposome solution were added.

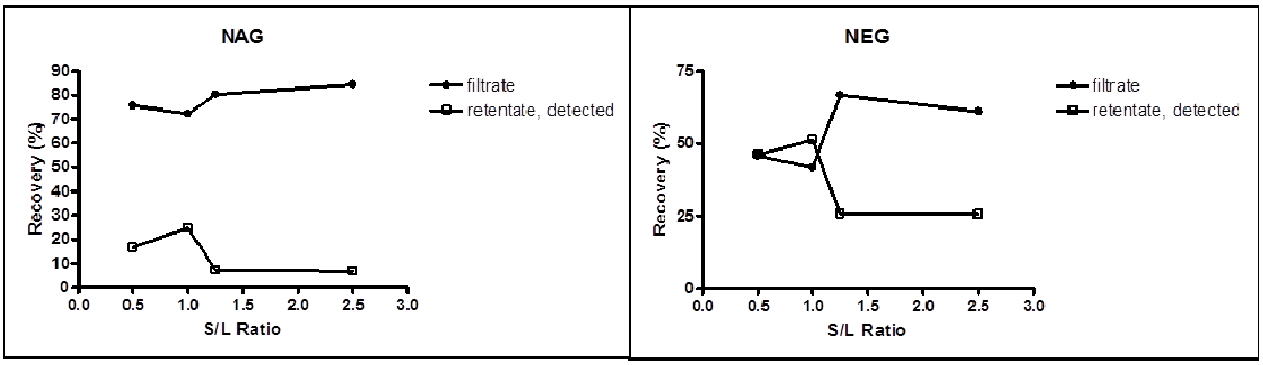

Figure 3: The decrease of NAG and NEG recovery caused by interaction with liposome (in this case EL-01-A) resulted inversely proportional to the liposome/steroid. ratio.

Liposomes, particularly their monomeric constituents, resulted also responsible for a dramatic effect on NAG and NEG derivatization. Table 1 presents the percentage of urine samples analyzed for GC-MS detection of NAG and NEG that presented a complete derivatization (andro mono/bis ratio <10%) in the presence of liposomes. It is evident that that the nature of the extraction solvent strongly influenced the derivatization efficiency. In fact, when a relatively polar organic solvent, such as TBME, is used in the liquid/liquid extraction of NAG and NEG in presence of liposomes, a huge amount of phospholipid was extracted together with the analytes. As a consequence of this, the concentrations of derivatizing agent commonly utilized during GC-MS analysis of steroids in doping controls resulted to be not sufficient for almost of the sample (only 1.4% of the samples with efficient derivatization, see table below).

Extraction procedure for NAG and NEG	Samples analyzed (n)	Samples with efficient derivatization (%)
LLE, TBME		1,4
LLE, *n*-pentane	72	75,0
SPE + LLE, *n*-pentane		100,0

Due to the inefficient derivatization, the analysis cannot be considered valid. A partial solution for this problem resulted to be the substitution of TBME with a more apolar solvent such as *n*-pentane. In this case, 75% of the samples presented an efficient derivatization. If combined with solid phase extraction with C18 cartridges, used as a further clean-up step before LLE extraction, this procedure allowed obtaining complete derivatization regardless the presence of phospholipids and/or liposomes.

Future goals: analytical approaches for the detection of liposomes in biological fluids

Several analytical strategies can be proposed to detect liposome-based DDS in biological fluids and/or to minimize masking effects of liposomes.

Classical liposomes are essentially constituted of phospholipids and cholesterol that, being common endogenous constituents, are not suitable as marker for their detection in biological fluids. Anyway, since they are not even efficient DDS, it is realistic to exclude their use also for doping purposes. As already mentioned, stealth liposomes, containing PEG-derivatized phospholipid, are tipically used to get suitable DDS. In this case, PEG-derived phospholipids, particularly distearoylphosphoetanolamine (DSPE-PEG), the most used one, represents good analytical targets. A double strategy can be followed: in blood, it is possible to look for intact liposomes: a flow cytofluorimetry method based on PEG immunofluorescent labelling that allow to detect PEGilated liposomes after blood fortification has been developed and is currently under review [8]. Flow cytofluorimetry is a widespread technique in doping analysis, utilized by WADA laboratories for the detection of blood transfusions. In second instance, the detection of DSPE-PEG as a liposome breakdown product excreted in urines by a liquid chromatography-mass spectrometry method is also under development and, additionally, the urinary phospholipid profile has been already investigated[9].

Conclusions: are liposomes a real threat?

This work demonstrated that liposomes can have important implications on doping analysis. For the future, it is necessary to evaluate whereas it is possible for cheating athletes to get liposome encapsulated drugs and therefore evaluate if liposomes represent a real threat for sport. Several liposomal formulation containing anabolic steroids are already being marketed, although by non-pharmaceutical sellers, and easily available on Internet, consisting of transdermal gels or oral formulation with increased bioavailability [10,11]. Additionally, further liposomal formulations containing potential doping agent such as glucocorticoids [12], hemoglobin [13], or even IGF-1 gene [14], some of which currently under clinical development, are documented by scientific literature.

Moreover, several other types of nanocarrier-based DDS are under development by the scientific community. Example of these systems are polymeric nanocarriers (e.g.: cyclodextrins-, polilactide-based) or vectors for use in gene therapy such as virus [15-17].

It is suggested to put attention on these emerging pharmaceutical formulations for slow release based on nanocarriers, that could represent a dangerous threat for sport, but also an opportunity for new analytical targets for the detection of performance enhancing drugs used.

Acknowledgement

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