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Are liposomes masking agents? An in-progress study

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Introduction

The use of chelating agents, and especially liposomes, is reportedly increasing among athletes, according to the outcome of several antidoping investigations [1]. Liposomes are microscopic spheres with an aqueous core surrounded by one or more outer shells consisting of phospholipids arranged in a bilayer configuration when hydrated in an aqueous medium [2-3]. The ability of liposomes to encapsulate or interact with therapeutic agents successfully, to modify the physicochemical properties of specific drugs and the pharmacokinetics of the drug was the basis for the hypothesis that liposomes may be used as masking agents in sport doping. The masking effect can potentially be achieved both after iv or im administration and by direct addition of liposomes to the sample at the time of collection.

We focused our attention on the interaction between liposomes and androgenic anabolic steroids (AAS), following the hypothesis that such an interaction, if significant enough, could make the detection of prohibited steroids by the analytical procedures more problematic. The procedures normally followed by the anti-doping laboratories are based on GC-MS analysis, after enzymatic hydrolysis, of the corresponding TMS-derivatives.

We have considered different steroid hormones and their metabolites (testosterone, epitestosterone, nandrolone, androsterone, etiocholanolone, norandrosterone, noretiocholanolone) as free drugs, and various liposome preparations differ in nature (anionic, weak anionic, non ionic, cationic) and phospholipid content and several liposome formulations (both purified standards and pharmaceutical formulations) are commercially available in Italy).

Experimental Section

We evaluated the steroids recovery in water following the effect of incubation with liposomes.

Sample pretreatment

The determination of the steroids was carried out by GC-MS after a pretreatment procedure, following the most common system for the detection of AAS currently followed by WADA-accredited anti-doping laboratories. The analytes were extracted using n-hexane at pH 7.4 and prior to GC-MS analysis the residues were derivatized with a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)/NH_4I/dithioerythreitol (1000:2:4 v/w/w) (see Figure 1). In order to check the extraction and derivatization steps, the corresponding deuterated steroids (testosterone D3, epitestosterone D3, nandrolone D3, androsterone D4, etiocholanolone D5, norandrosterone D4, noretiocholanolone D4), as free standards, were added to the organic phase, just before the derivatisation step, as reference compounds for the recovery calculation of the corresponding steroids; methyltestosterone was added at the same time as chromatographic reference standard and androsterone mono-TMS and androsterone D4 mono-TMS ions were assessed as derivatization target compounds. The blank trials were performed under the same experimental conditions except for the addition of liposomes.

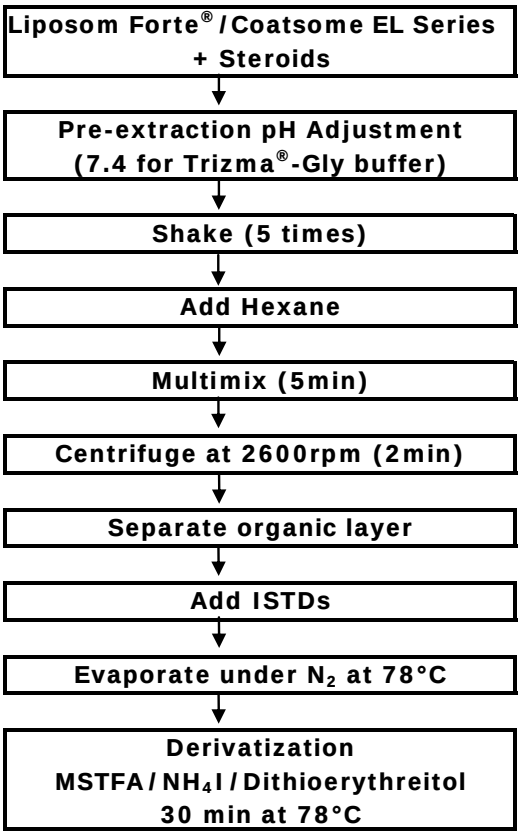

Figure 1. Flow chart of the sample pretreatment.

GC-MS conditions

The trials were performed on a GC-MS (Hewlett Packard 5973 mass selective detector directly coupled to a Hewlett Packard 6890 gas chromatograph) using the following conditions: Column: capillary 100 % methylsilicone (l = 17 ±1 m, id = 0.20 mm, film thickness = 0.11 μm); T oven gradient: $T_{initial}$ = 188 °C, 2.5 min; ramp 3 °C/min to 211 °C, 2 min; ramp 10 °C/min to 238 °C, ramp 40 °C/min to 320 °C; 3 min at 320 °C; V_{inj} = 1 mL; split mode (split ratio: 1/10; split flow: 5.7 mL/min); $T_{injector}$ = 280 °C; $T_{transfer\ line}$ = 280 °C; Carrier gas: He at P=const flow (0.6 mL/min); average velocity: 39 cm/sec; Electron Impact MS detector; SIM acquisition.

Data evaluation

The robustness of the method and the repeatability of the quantitative determinations were evaluated with good results. All the assays were performed at least in duplicate.

Recoveries were calculated as:

$$rec\% = \frac{\left(area_X / area_{deutX}\right)_{liposomes}}{\left(area_X / area_{deutX}\right)_{blank}} \cdot 100$$

Results and Discussion

Since the use of substances and methods able to alter the normal pharmacokinetics of drugs is reportedly increasing among athletes, we postulated that some of these substances (and primarily among them liposomes) can interact with other "doping" substances (primarily among them steroidal compounds), making their detection by the antidoping laboratories more problematic. This can be the consequence of a reduction of their free circulating concentrations and, in turn, a slower urinary excretion (a putative masking strategy based on the theoretical approach of controlled-release drug formulations) and/or of a reduction of their recovery in urine, due to an interference with the analytical procedures followed for the detection of anabolic steroids. This interaction could promote the use of liposomes as masking agents by following one or more of the following hypothesized masking strategies:

1. Injection immediately after being mixed with steroids, to produce "home-made" slow/sustained release preparations;
2. Injection as such ("empty"), before an "expected" anti-doping test, to promote the interaction with the steroids/metabolites circulating in the organism and therefore altering their excretion profile;
3. Direct addition to the sample collected for the anti-doping test, to reduce the concentration of "free" (i.e. not bound to liposomes) steroids/metabolites, and therefore reducing the efficacy of the analytical procedures used for their detection.

The masking effect can potentially be achieved both after intravenous or intramuscular administration and/or by direct addition of liposomes to the sample at the time of collection. We focused on their interaction with one of most frequently abused classes of prohibited substance in sport doping, androgenic anabolic steroids (AAS). AAS continue to be reported as the most commonly abused class of prohibited substances. In 2008, WADA reported that 59.0 % of all adverse analytical findings were found to be anabolic agents [4]. This class of banned substances includes mainly AAS as well as other anabolic agents.

The capability of liposomes to interact with steroids was found to depend on the following factors: input concentration, hydrophobicity, aqueous solubility, and molecular weight of the steroids [5]. The efficiency of the interaction increases with increasing drug input, hydrophobicity, and molecular weight [6]. The interaction becomes less efficient with increasing aqueous solubility of the steroids; the hydrophobicity of steroids improves their interaction with liposomes. Lipophilicity is so beneficial for liposomal interactions that it has been exploited by means of lipophilic derivatization of drug molecules to increase liposomal interaction [7]. Another reason anabolic steroids may interact so successfully with liposomes is because of their structural similarity to a possible bilayer component, cholesterol [6]. It is therefore reasonable to postulate that, although in a limited range of experimental conditions, the concentration of "available" steroid would be reduced in the presence of liposomes.

We tested the capability of liposomes to interact with free (nonconjugated) AAS in buffered standard solutions. The most common system for the detection of AAS currently followed by the majority of WADA-accredited anti-doping laboratories involves the use of solid-phase or liquid-liquid extraction of the urine sample followed by a derivatization step and then analysis by gas chromatography-mass spectrometry (GC-MS). We have specifically investigated all those stages of the analytical procedure (and especially extraction and derivatization) whose yield could, in principle, be altered in the presence of liposomes.

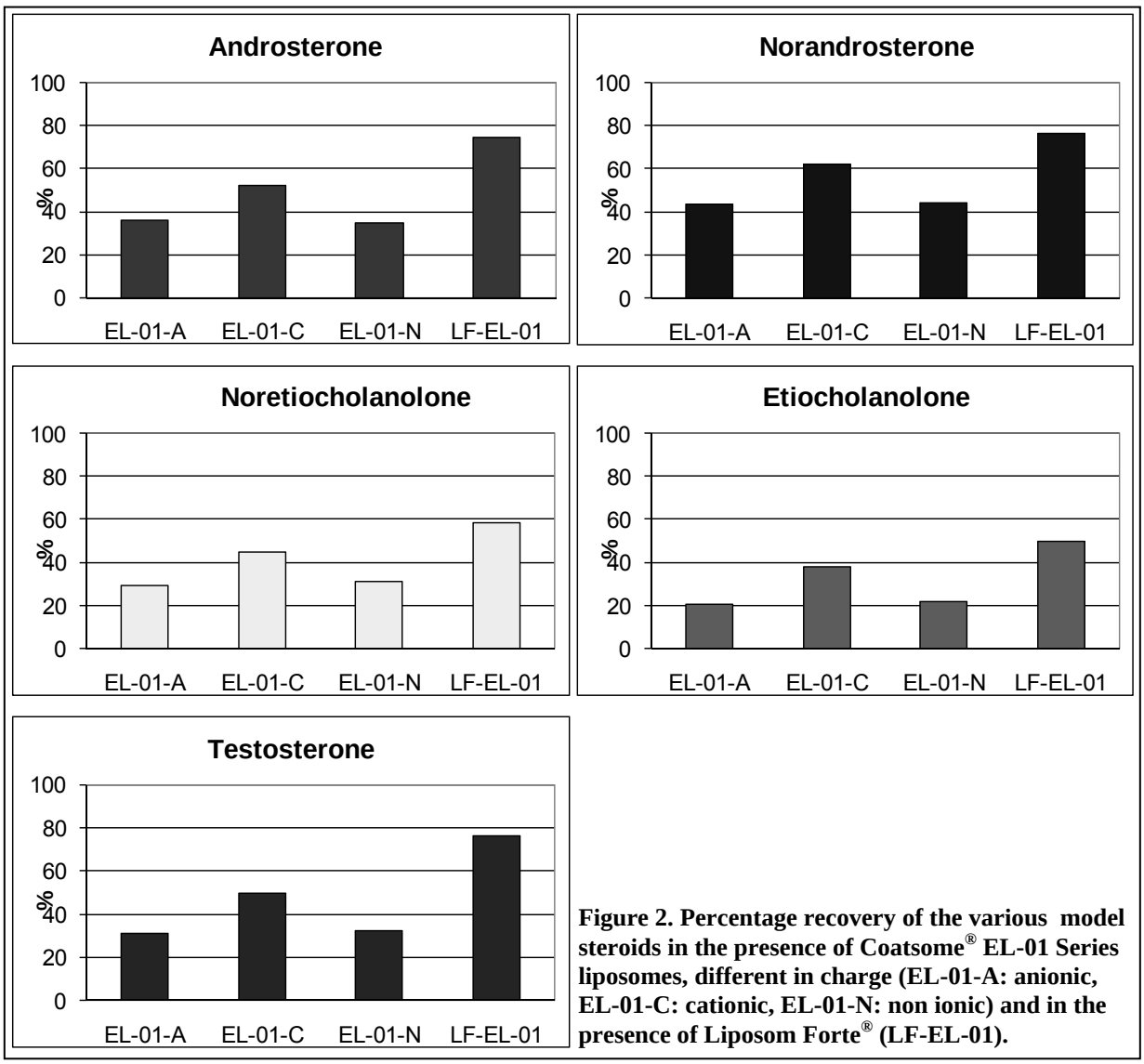

Figure 2. Percentage recovery of the various model steroids in the presence of Coatsome® EL-01 Series liposomes, different in charge (EL-01-A: anionic, EL-01-C: cationic, EL-01-N: non ionic) and in the presence of Liposom Forte® (LF-EL-01).

We have considered different model steroid hormones and their metabolites (testosterone, epitestosterone, nandrolone, androsterone, etiocholanolone, norandrosterone, noretiocholanolone) in the free (nonconjugated) form. We have also assessed various liposome preparations including those that differ in charge (anionic, slightly anionic, non ionic, cationic), differ in phospholipid content, and have varying liposome formulations (both purified standards and pharmaceutical formulations such as "Liposom Forte®," which is commercially available in Italy).

Steroid	EL-01-A	EL-01-C	EL-01-N	LF-EL-01
Testosterone	30-32	48-50	30-34	74-80
Androsterone	34-37	49-52	32-35	72-75
Etiocholanolone	19-23	36-40	20-23	48-50
Norandrosterone	40-45	60-62	42-45	75-78
Noretiocholanolone	28-32	43-46	29-32	55-60

Table 1. Percentage recovery of the various model steroids in the presence of Coatsome® EL-01 Series liposomes, different in charge (EL-01-A: anionic, EL-01-C: cationic, EL-01-N: non ionic) and in the presence of Liposom Forte® (LF-EL-01). Average of five trials, each performed in triplicate.

We evaluated the recovery of free steroids in buffered solutions at different pH and in the presence of different buffering systems. Recovery was evaluated after sample preparation and steroid extraction either in the presence or in the absence of liposomes. The recovery, measured from a nominal concentration of 250 ng/mL, was decreased to 20 % in the presence of liposomes (see Table 1). The reduction in measured steroid concentrations in the presence of liposomes followed the order: etiocholanolone ≥ noretiocholanolone > androsterone ≥ norandrosterone ≥ nandrolone = testosterone ≥ epitestosterone (see Figure 2). The effect of the phospholipid composition of liposomes, as well as the liposome nature, affected the percentage of recovery (see Figure 3). The reduction of the recovery following the order anionic ≥ slightly anionic = non ionic > cationic, and with commercial liposomes giving the same results of cationic liposomes (see Figure 4).

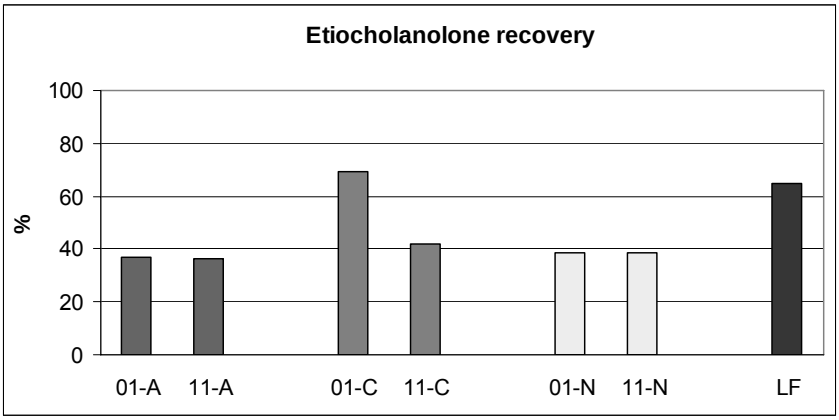

Figure 3. Percentage recovery of etiocholanolone (as an example) in the presence of Coatsome® EL-01 and EL-11 Series liposomes, different in composition (EL-01: di-palmitoyl phosphatidylcholine, cholesterol, di-palmitoyl phosphatidylglycerol (A, N)/ stearyl amine (C) ; EL-11: palmitoyl oleoyl phosphatidylcholine, cholesterol, palmitoyl oleoyl phosphatidylglycerol (A, N)/ stearyl amine (C)), and in the presence of Liposom Forte® (LF).

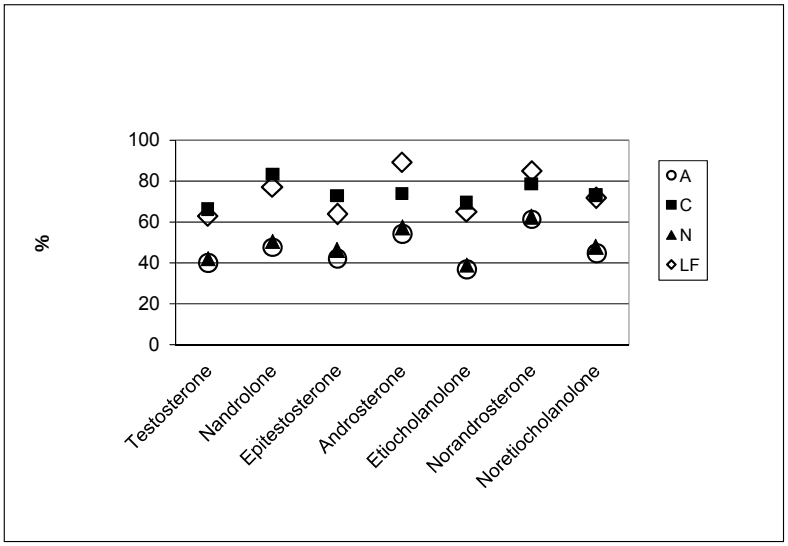

Figure 4. The trend of steroid recovery with pure liposomes different in charge (Coatsome® EL-01 Series; A: anionic, C: cationic, N: non ionic) and with commercial liposomes (Liposom Forte®; LF). Average of six trials, each performed in triplicate.

The measured effects, although reproducible in a relatively wide range of experimental conditions, seem to depend on the pH value and on both the nature and the concentration of the buffer system used (see Figure 5).

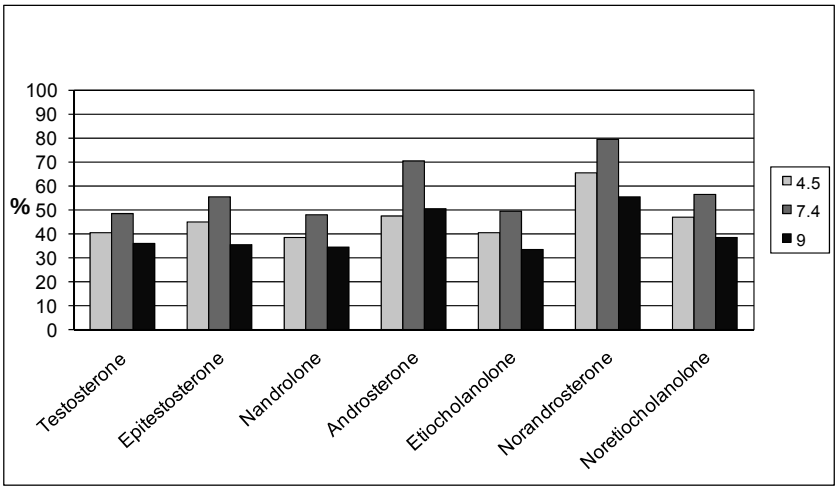

Figure 5. Extraction pH effect on steroid recovery after liposome-steroid interaction in water (Coatsome EL-01-A Series, anionic). Percentage recovery in acid (pH 4.5 for formic acid), neutral (pH 7.4 for Trizma™-Gly buffer) and alkali (pH 9.0 for ammonium hydroxide).

Conclusions, Current Studies and Future Perspectives

We have preliminarily shown that an interaction between liposomes and androgenic anabolic steroids occurs. This interaction can cause a reduced efficacy of the analytical procedures used; the amount of steroid detected in the samples was significantly reduced after liposome addition.

Currently in progress are recovery trials on spiked urines, carried out to assess the masking potential of liposomes on threshold steroids, like 19-norandrosterone. We also plan to assess the effect liposomes have on additional steroids, representative of the most common classes of synthetic anabolic-androgenic steroids (e.g. 19-norsteroids, 17α-methylated steroids and A-ring modified steroids) in a similar manner, as well as their glucuronide conjugates in varying concentrations and liposomes in varying amounts fortified into urine. Similarly, these steroids and liposomes will be assessed after their fortification into blood.

Following the study of the interaction between AAS and liposomes *in vitro*, we will assess the masking potential of liposomes *in vivo* and finally we plan to setup a method for the detection of synthetic liposomes and/or their components in the body fluids used for anti-doping analysis.

Acknowledgement

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