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A rapid analytical method for the detection of plasma volume expanders and mannitol based on the urinary saccharides profile

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Introduction

The misuse of plasma volume expanders (PVE) in sport is prohibited according to the World Anti-Doping Agency list of prohibited substances and methods [1]. Athletes could illicitly use dextran (DES) and hydroxyethyl starch (HES) for three main reasons: (i) to keep under control their haematocrit or haemoglobin values, following illicit stimulation of erythropoiesis, (ii) to enhance microcirculation, leading to an improved oxygen transport to tissues and muscles, and (iii) to increase body fluid amounts, re-balancing the loss of liquids due to dehydratation that could cause a reduction in sport performance [2-3]. Whereas mannitol, an osmodiuretic agent, with well defined uses in clinical pharmacology could be misused for its capability to decrease the body-weight and to dilute the urine with consequent reduction of the urinary levels of other banned substances [4]. Here we present a screening procedure developed for the detection of glucose, isomaltose, maltose, hydroxyl-ethylated maltose, sucrose, mannitol and sorbitol in human urine. The proposed analytical procedure, which involves only one enzymatic hydrolysis step and the direct injection into a LC-MS/MS system, was set up and validated to detect the abuse of mannitol and of the PVEs, dextran and HES in doping analysis. The developed method was validated and applied to estimate the reference urinary concentration ranges of the compounds here considered in control samples, with the aim to select the most appropriate marker of HES, dextran and mannitol abuse, to be used as analytical target in doping analysis.

Experimental

The 6% HES solution (Voluven®) and the 5% dextran solution (Plander®) were from Fresenius Kabi (Verona, Italy). Glucose-$^{13}C_6$ (used as internal standard), glucose, isomaltose, maltose, mannitol, sorbitol, sucrose, the enzymes dextranase from *Penicillium sp*. (used for dextran hydrolysis) and α-amylase from human saliva (Type IX-A) (used for HES

hydrolysis) and all chemicals were from Sigma-Aldrich (Milan, Italy). Mannitol-$^{13}C_1$ (used as internal standard) was from Cambridge Isotope Laboratories, Inc. (MA, USA). The ultrapure water used was of Milli-Q-grade (Millipore, Milan, Italy). The artificial urine (used to evaluate the lower limit of detection (LLOD) and quantification (LLOQ), accuracy, linearity and precision) was prepared following the protocol described by Leinonen *et al* [5].

A total of 330 urine samples (300 from routine doping control tests and 30 from healthy volunteers) were analyzed for the presence of glucose, isomaltose, maltose, hydroxyl-ethylated maltose, mannitol, and sucrose. All LC-MS/MS experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump and a Varian Polaris 5 NH2 (150 X 2.1 mm, 5 µm) column (Agilent Technologies S.p.A, Milan, Italy). The mobile phase was 5 mM ammonium formate, 0.1% formic acid (A) and acetonitrile (B). The gradient program started at 20% A and increasing to 50% A in 11 min. The column was finally re-equilibrated at 20% A for 2 min. The flow rate and the column temperature were set at 250 µL/min and 40 °C respectively. Mass spectrometric detection was performed using an API4000 triple-quadrupole instrument (Applera Italia, Monza, Italy) with negative electrospray ionization. The ion source was operated at 450 °C, the applied capillary voltage was -4500 V, the declustering potential was set at -60 V and SRM experiments were performed (Table 1).

Table 1: Ion transitions, retention times (Rt), LLOD and LLOQ

Compound	**Ion Transitions (*m/z*)**	**Rt (min)**	**LLOD/LLOQ (µg/mL)**
Glucose-$^{13}C_6$ (ISTD1)	231/185; 185/123; <u>185/92</u>	4.73/5.09	---
Glucose (GLU)	225/179; 225/119; <u>225/89</u>	4.74/5.10	50/100
Isomaltose (ISO)	387/341; <u>387/251</u>; 387/161	9.21/9.91	30/100
Maltose (MALT)	387/341; 387/221; <u>387/161</u>	7.90/8.48	50/100
Maltose hydroxy-ethylated	431/385; 431/223; <u>431/161</u>	6.04-8.37*	---**
Mannitol-$^{13}C_1$ (ISTD2)	228/182; <u>228/90</u>	5.26	---
Mannitol (MAN)	<u>227/181</u>; 227/163; 227/119	5.06	30/70
Sorbitol (SORB)	<u>227/181</u>; 227/163; 227/119	5.06	30/70
Sucrose (SUCR)	<u>387/341</u>; 387/179; 387/161	7.03	30/70

Underscored transitions were used for quantification
* Multiple peaks were detected due to the presence of hydroxy-ethyl groups on C 2 or 3 or 6 of the glucose backbone
** LLOD and LLOQ not calculated due to the lack of reference compounds

To 40 µL of urine, 80 µL of phosphate buffer (2 mM, pH 6), 10 µL of α-amylase from human saliva (1 mg/mL; 210 units/mg) and 10 µL of dextranase (1.5 mg/mL, 25.3 units/mg) were added and the samples were incubated for 60 min at 37 °C. After hydrolysis 20 µL of the ISTDs mixture (Mannitol-$^{13}C_1$ and Glucose-$^{13}C_6$ at a final concentration of 300 µg/mL) were added and the samples were evaporated to dryness under N_2 stream at 70 °C. The

residue was reconstituted with 20 µL of distilled water and 100 µL of acetonitrile and an aliquot of 10 µL was injected into the LC-MS/MS system.

Results and Discussion

The selected chromatographic conditions allowed the efficient separation of all the compounds with the same molecular weight with the exception of MAN and SORB, which are eluted at the same retention time and share the same mass spectrum (see Figure 1).

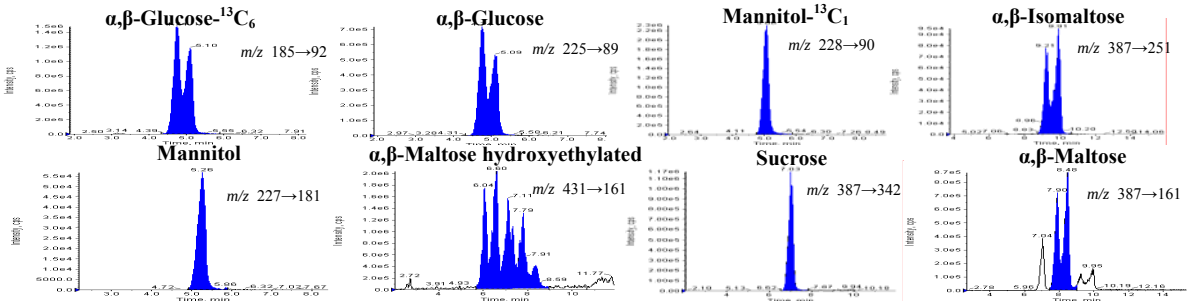

Figure 1: Standard mixture of HES (1000 µg/mL) and GLU, GLU-$^{13}C_6$, ISO, MAN, MAN-$^{13}C_1$ and SUCR (300 µg/mL) after enzymatic hydrolysis.

Good linearity (R^2 0.990-0.995), precision of peak areas (with variability not exceeding 10% for intra-day assays and 15% for inter-day assays at 200, 500 and 1000 µg/mL concentration levels using ten replicate each), and reproducibility of the relative retention times (CV% < 1) and of relative abundances of selected ion transitions (CV% < 10), were assessed. Carryover problems were not experienced in blank samples injected just after the analysis of the fortified artificial urine at five times the LLOQ concentrations. A suppression of ESI responses (< 30%) was observed at the Rts of the target analytes and ISTDs while 20 different blank urine samples were injected. Concerning the specificity, the identity of the target compounds was characterized by liquid chromatographic retention times and by the product ion spectra in 20 blank urines: no other compounds interfered with the target analytes or the ISTDs. The LLOD and LLOQ were in the range of 30-100 µg/mL. The deviation of the mean measured concentration from the theoretical concentration was below 15% for all the three levels tested (200, 500 and 1000 µg/mL) using ten replicates each.

The study of the normal urinary ranges of the saccharides and polyalcohols here considered in 330 urine samples showed that the best markers of HES and DES abuse are the hydroxyl-ethylated maltose and ISO respectively, whereas for MAN a threshold value might be established in order to enable doping control laboratories to report abnormal analytical findings (Figures 2A-B). The MAN levels were not influenced by the co-elution with SORB because its physiological concentrations is lower than the LLOQ of the developed procedure.

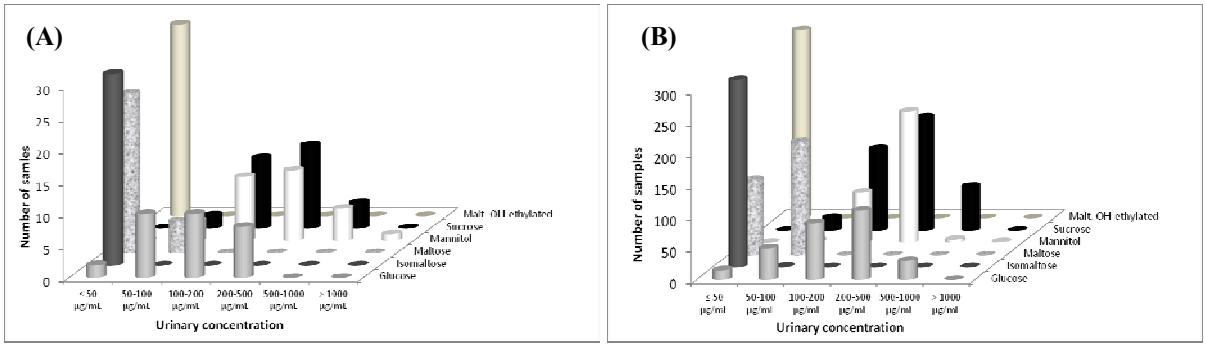

Figure 2: Saccharides and MAN physiological ranges in 30 urine samples from healthy volunteers **(A)** and in 300 routine doping control samples **(B).**

In summary this study presents a novel approach, involving a single enzymatic hydrolysis step and the injection into the LC-MS/MS system, to detect in a single run urinary markers of the abuse of DES, HES and MAN. The analytical procedure is longer compared to the recently proposed strategies [6-7] to screen DES and HES using in-source collision-induced dissociation. Nevertheless, thanks to the high selectivity and specificity and to the presence of more than three product ions higher than 10% in the product ion spectra of maltose hydroxyl-ethylated and ISO (data not shown) it could be used also as a final confirmation method, avoiding the need of additional, complex confirmation procedures being the urinary levels of HES, DES and MAN after intravenous administration higher than 5 mg/mL for more than 24 h as reported by previous investigators [3,5,8].

References

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