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

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A screening procedure specifically developed for the detection of saccharides and polyalcohols in human urine in the framework of doping control analysis is presented. The proposed method, set-up, and validated to detect the abuse of dextran, hydroxyethyl starch and mannitol as a doping practice in sport, involves only one enzymatic hydrolysis step and the direct injection into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

The chromatographic conditions were optimized to allow the efficient separation of compounds with the same molecular weight. Good linearity (R^2 0.990–0.995) and reproducibility of relative retention times (CV% lower than 1) and of relative abundances of characteristic ion transitions (CV% lower than 10) were obtained. The lower limits of detection and quantification were in the range of 30–100 μg/ml.

Since the analytes studied are present also in non-doping products (e.g. in fruit as well as in food products and drugs additives), the developed method was also used to establish a range of reference urinary concentrations: 600 doping control samples and 30 samples from volunteers not using any medication were considered. While the hydrolysis products (isomaltose and maltose hydroxyl-ethylated), used as specific markers for the detection of dextran and hydroxyethyl starch abuse, were not detected in urine; mannitol was present in all urines in a concentration range of 30–1200 μg/ml. Since no criteria of positivity for mannitol has been established yet, the results obtained in this study could be considered, in combination with those of previous researches, as a starting point to fix a threshold value for doping control purpose. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: anti-doping analysis; mannitol; masking agents; polysaccharide-based plasma volume expander

Introduction

The misuse of plasma volume expanders (PVE, i.e. mannitol, dextran and hydroxyethyl starch) in sports is prohibited according to the World Anti-Doping Agency (WADA) list of prohibited substances and methods. They are included with other similar drugs in Section 5: Diuretics and other masking agents.[1]

Athletes could illicitly use dextran and hydroxyethyl starch (HES) for three main reasons: (1) to keep under control their haematocrit or haemoglobin values, following illicit stimulation of erythropoiesis; (2) to enhance microcirculation, leading to better oxygen transport into tissue and muscle; and (3) to increase body fluid amounts, re-balancing the loss of liquids due to dehydratation that could cause a reduction in sport performance.[2–6] Whereas mannitol, an osmodiuretic agent, with well-defined uses in clinical pharmacology (nowadays limited to the following: (1) reduction of cerebral oedema and brain mass before and after neurosurgery, (2) treatment of acute tubular necrosis as a renal protector and for the treatment of dialysis, and (3) control intraocular pressure during acute attacks of glaucoma and in ocular surgery) could be misused for its capability to decrease the body-weight and to dilute the urine with consequent reduction of the urinary concentration of other banned substances.[7,8]

The structure of HES consists of D-glucose units linked via linear α-1,4-bonds in which the number of α-1,6 branch points can vary. Hydroxyethyl groups are attached to carbons 2 (70%), 3 (20%) or 6 (10%) of the glucose units according to the different reactivity of the hydroxyl groups. The degree of substitution is variable, depending on the specific formulations presently available on the market, and varies from 35 up to 70%. Its average molecular weight in remedies varies from 60 000 to 130 000 Dalton and it is usually administered as a 6% solution in 0.9% aqueous NaCl.[2,3] Dextran is a polysaccharide of 1, 6-linked α-D-glucose units without any substitution and a branching degree of 7% at the position C-3 and C-4. Its average molecular weight in remedies varies from 40 000 to 70 000 Dalton and it is usually administered as a 5–10% solution in 0.9% aqueous NaCl.[8] Finally mannitol is an hexahydric sugar alcohol usually administered as a 10–20% solution.[7]

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Mannitol is freely filtered through the renal glomeruli,[7] whereas HES and dextran have to be cleaved into smaller units to pass the renal barrier.[2,3,9–12] Specifically, the metabolism of HES proceeds through α-amylase hydrolysis of glycosidic bonds, but does not result in complete degradation due to the hydroxyethylation of several glucose residues.[2,3] The hydrolysis of dextran is instead dependent on cell-bound enzymes, due to the lack of plasma enzymes able to cut 1,6-linked glucose.[9]

Analytical screening procedures applied by the anti-doping laboratories to detect dextran, HES and mannitol in human urine are based on either gas chromatography-mass spectrometry (GC-MS)[2,3,7,13] and/or liquid chromatography-tandem mass spectrometry (LC-MS/MS).[5,9,14,15] Most of these methods require toxic reagents (i.e. pyridine, methyl iodide) and time-consuming pretreatment steps (an acid or enzymatic hydrolysis to degrade/digest the polysaccharides to glucose, hydroxyethylated glucose, isomaltose, maltose or hydroxyethylated maltose and one or more derivatization steps). To speed up the dextran and HES screening analysis, different kinds of colourimetric and electrochemical methods have been proposed by previous investigators.[16–23] Nonetheless, mass spectrometric approaches seem to remain as the methods of choice due to their high specificity. Here we present an analytical screening procedure, involving only one enzymatic hydrolysis step and the direct injection into the LC-MS/MS system, specifically developed to detect, in a single run, the intake of dextran, HESl and mannitol in human urine. This method was validated according to ISO 17025[24] and to the WADA requirements for the accredited laboratories (as detailed in the WADA International Standard for Laboratories and related technical documents[25,26]) and applied to estimate the saccharides (glucose, isomaltose, maltose, maltose hydroxyl-ethylated, and sucrose) and polyalcohols (mannitol and sorbitol) physiological urinary concentration ranges in 600 doping control samples and in 30 samples from healthy volunteers not using any medication, with the aim to select the most appropriate marker of HES, dextran and mannitol abuse and to suggest criteria of positivity for those compounds included in the WADA list and normally present in human urine.

Experimental

Chemicals and reagents

The 6% hydroxyethyl starch (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 and sucrose were from Sigma-Aldrich (Milan, Italy). Mannitol-$^{13}C_1$ (used as internal standard) was from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

HES and dextran excretion studies samples were supplied by the accredited sports anti-doping laboratory of Cologne (Germany).

The enzymes dextranase from *Penicillium sp*. (used for the hydrolysis of dextran) and α-amylase from human saliva (Type IX-A) and from *Bacillus sp*. (Type IIA) (enzymes tested for the hydrolysis of HES) were from Sigma-Aldrich (Milan, Italy).

All chemicals (acetonitrile, formic acid, sodium phosphate, sodium hydrogen phosphate, ammonium formate, urea, ammonium phosphate, alanine, glycine, creatinine, bovine albumine, sodium chloride, oxalic acid and citric acid monohydrate) were provided by Sigma-Aldrich (Milan, Italy). The ultrapure water used was of Milli-Q-grade (Millipore, Milan, Italy).

Mannitol, glucose, isomaltose, maltose, sorbitol, sucrose, HES and dextran water standard solutions were prepared at 1 mg/ml with 0.1% of acetonitrile to avoid the potential bacterial degradation of the analytes and stored at 4 °C for a maximum of 1 month.

The artificial urine was prepared following the protocol described by Leinonen *et al*.[27]

Urine samples

A total of 630 urine samples were analyzed for the presence of glucose, isomaltose, maltose, maltose hydroxyl-ethylated, mannitol, and sucrose. Six hundred samples were from regular doping control urines tested negative in all screening procedures and derived from in-competition (300 samples) as well as out-of-competition (300 samples) testing of different sports disciplines (i.e. cycling, basketball, football, swimming, rugby and volleyball). Finally, 30 urine samples were collected from healthy volunteers not using any medication and showing negative results in all screening procedures.

Sample preparation

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 internal standards 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 N2 stream at 70 °C. The residue was reconstituted with 20 µl of distilled water and 100 µl of acetonitrile. Then an aliquot of 10 µl was injected into the LC-MS/MS system.

Instrumental conditions

All LC-MS/MS experiments were performed using an Agilent 1200 Rapid Resolution Series high performance liquid chromatography (HPLC) pump with binary gradient system and automatic injector (Agilent Technologies S.p.A, Cernusco sul Naviglio, Milan, Italy). LC was performed using a Polaris 5 NH2 (150 X 2.1 mm, 5 µm) column from Varian. The mobile phase used was 5 mM ammonium formiate, 0.1% formic acid (A) and acetonitrile containing 0.1% of formic acid (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 was set at 250 µl/min. The column temperature was set at 40 °C.

Mass spectrometry was performed using an Applied Biosystems (Applera Italia, Monza, Italy) API4000 triple-quadrupole instrument with negative electrospray ionization (ESI). The ion source was operated at 450 °C, the applied capillary voltage was −4500 V and selected reaction monitoring (SRM) experiments were performed employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8 mPa, obtained from a dedicated nitrogen generator system Parker-Balston model 75-A74, gas purity 99.5% (CPS analitica Milan, Italy). The collision energy (Table 1) and the declustering potential (set at −60 V) were optimized by the direct infusion of standard solutions of each analyte at a concentration of 300 µg/ml. Analyst™ software, Ver. 1.5.1 (Applied Biosystems / MDS Sciex) is used for data acquisition and processing.

Assay validation

Experiments were performed using artificial urine to determine the lower limits of detection and quantification, robustness, linearity, intra- and inter-day precision and accuracy; whereas for the determination of specificity, ion suppression/enhancement

Table 1. Mass spectrometer parameters, retention times, LLODs and LLOQs of the compounds considered in this study

Compounds	SRM Transitions (*m/z*)	Collision Energy (eV)	Retention Time (min)	LLOD/LLOQ (μg/ml)
Glucose-$^{13}C_6$ (ISTD1)	231/185; 185/123; 185/92	−30; -35; -40	4.73/5.09	---
Glucose	225/179; 225/119; 225/89	−30; -35; -45	4.74/5.10	50/100
Isomaltose	387/341; 387/251; 387/221; 387/161	−15; -20; -30; -40	9.21/9.91	30/100
Maltose	387/341; 387/221; 387/161;	−15; -25; -40;	7.90/8.48	50/100
Maltose hydroxy-ethylated	431/385; 431/223; 431/161	−15; -25; -35;	6.04-8.37*	---**
Mannitol-$^{13}C_1$ (ISTD2)	228/182; 228/90	−15; -40	5.26	---
Mannitol	227/181; 227/163; 227/119; 227/89	−15; -35; -40; -45	5.06	30/70
Sorbitol	227/181; 227/163; 227/119; 227/89	−15; -35; -40; -45	5.06	30/70
Sucrose	387/341; 387/179; 387/161	−15; -45; -45	7.03	30/70

Underscored ion transitions were used for quantification.

* More than one peak was detected at the retention time of maltose hydroxyl-ethylated due to the presence of hydroxy-ethyl groups attached to carbons 2 or 3 or 6 of the glucose units.

** The measurement of LLOD and LLOQ for the maltose hydroxyl-ethylated was not possible due to the lack of individual reference compounds.

and relative abundances of characteristic ion transitions and retention time repeatabilities at least 20 blank urines were utilized.

For the determination of the lower limit of detection (LLOD) and quantification (LLOQ) at least 10 aliquots of artificial urine spiked with the compounds here considered at a concentration of 500 μg/ml were used. Serial 1:2 dilutions were made and the LLOD was reported as the lowest concentration at which a compound could be identified in all 10 aliquots tested with the least abundant diagnostic ion transition observed with a signal-to-noise (S/N) ratio greater than 3. The LLOQ was reported as the lowest measured concentration that can be determined in a reproducible way with an S/N greater than 9.

The effect of the urine matrix on ion suppression/enhancement was assessed according to established protocols[28,29] by post-column continuous infusion of the hydrolyzed HES solution (500 μg/ml) and of the deuterated analytes (Mannitol-$^{13}C_1$ and Glucose-$^{13}C_6$), and isomaltose reference compounds at a concentration of 300 μg/ml via a syringe pump (flow rate of 7 μl/min) and observation of the ESI response during injection of 20 different urine specimens into the LC-MS/MS system.

The retention time and the relative abundances of characteristic ion transitions repeatabilities were evaluated from the intra-assay variations in 20 different blank urines spiked with all compounds considered at a concentration of 300 μg/ml and analysed through the procedure.

To evaluate the specificity at least 20 different urines were prepared as described to probe for interfering peaks in the selected ion chromatograms and product ion chromatograms at the expected retention times for the analytes and internal standards considered in this study.

The robustness was demonstrated by analyzing 10 aliquots of artificial urine spiked with the compounds studied at a concentration five times the LLOD, once a week for seven weeks, randomly changing the instrument and the operator involved in the instrumental analysis and in the preparation of the urine samples.

For the linearity a calibration curve was obtained fortifying different aliquots of artificial urine with all the compounds studied at six concentration levels (100, 200, 300, 500, 1000, and 2000 μg/ml) prepared in triplicate. Averages of the triplicate were used to construct the calibration curve. The area ratios between each compound and the internal standards (Mannitol-$^{13}C_1$ for mannitol and sucrose and Glucose-$^{13}C_6$ for glucose, isomaltose and maltose, both at a final concentration of 300 μg/ml) were plotted versus the concentrations. Curves showing a quadratic regression coefficient (R^2) higher than 0.99 were considered satisfactory.

Intra- and inter-day precision were measured in at least 10 aliquots of the artificial urine spiked at three different concentrations: low (200 μg/ml), medium (500 μg/ml) and high (1000 μg/ml) processed during the evaluation of the robustness of the method.

The accuracy was studied at three levels (200, 500, and 1000 μg/ml) in at least ten aliquots prepared and analyzed at least six times. The accuracy was expressed as the percentage deviation of the mean calculated value (obtained using an external calibration curve) from the nominal sample concentration. Maximal tolerance is +/− 15%.

Results and discussion

Mass spectrometric conditions

The standard solution of each analyte considered in this study at a concentration of 300 μg/ml was infused in positive and negative ESI in order to study the ionization behaviour. All compounds were detected as Na^+ adducts in positive ionization and as formiate (or acetate when the mobile phase additives are acetic acid and ammonium acetate) adducts in negative ionization. Nonetheless, the product ion spectra obtained in positive ESI, at low and high collision energies, contain few diagnostic ions: the monomer ($[M + Na]^+$) and the dimer ($[2\,M + Na]^+$) ions with sodium (data not shown). On the contrary, as shown in Figures 1A and 1B, the mass spectra obtained using negative ionization are very informative. Specifically, at low collision energy (lower than −20 eV) the most abundant ions observed were the formiate adduct and the $[M\text{-}H]^-$ ion resulting from the loss of the formiate; whereas increasing the collision energy (higher than −20 eV) water (−18 Da), formaldehyde (−30 Da) and acetaldehyde (−44 Da) losses were observed in all product ion spectra. Furthermore in the product ion spectra of isomaltose, maltose, maltose hydroxyethylated and sucrose the loss of glucose-H_2O (−162 Da) from the precursor ion at *m/z* 341 (isomaltose, maltose and sucrose) and at *m/z* 385 (maltose hydroxyl-ethylated) was detected. Finally in the product ion spectrum of the maltose hydroxyl-ethylated the loss of the hydroxyl-ethylated moiety (−62 Da) from the precursor ion at *m/z* 223 was also observed.

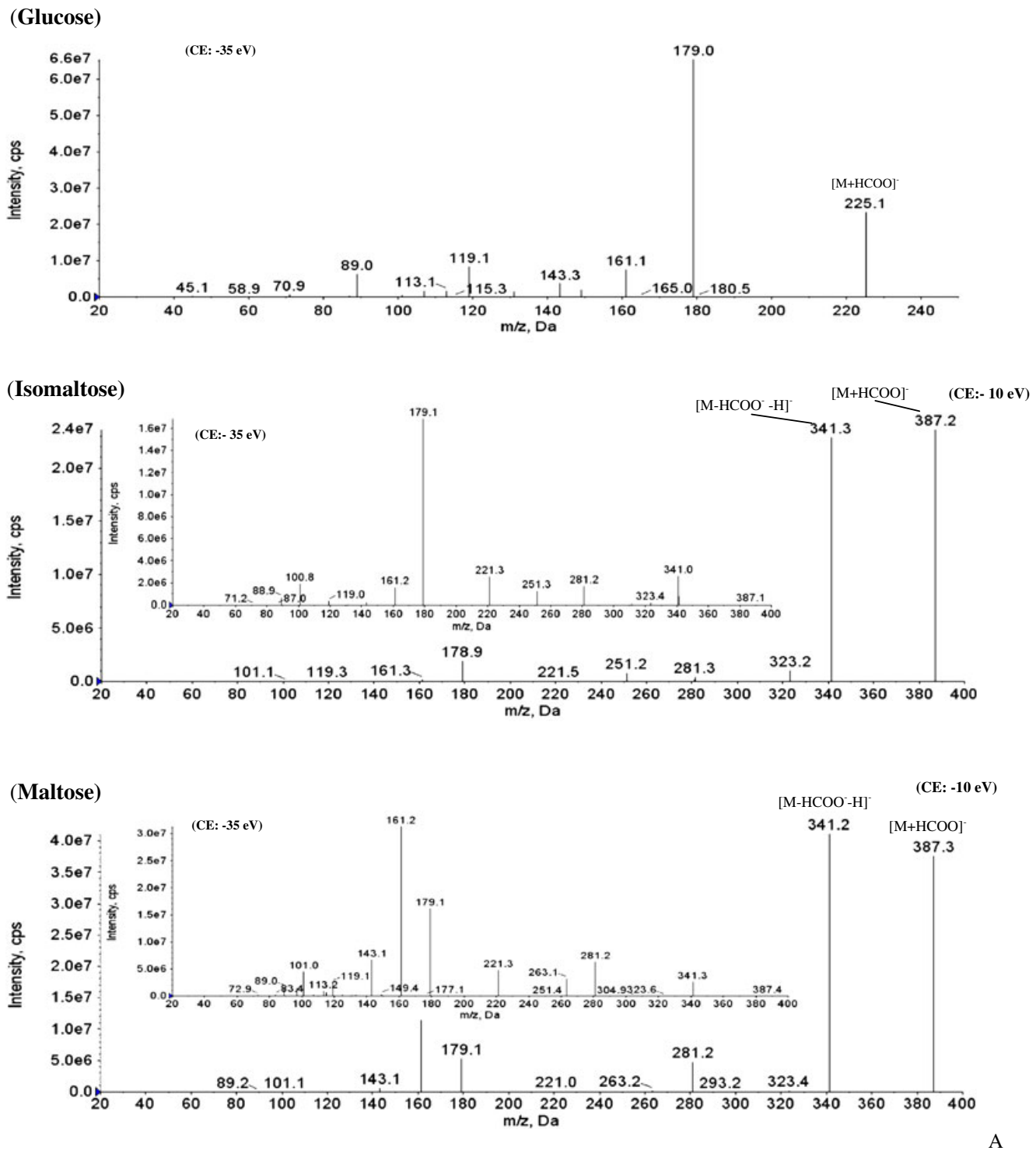

Figure 1. **(A)** Product ion spectra of glucose, maltose and isomaltose obtained at two different collision energies (−10 and −35 eV) using the formiate adduct (*m/z* 225 for glucose and *m/z* 387 for isomaltose and maltose) as parent mass. **(B)** Product ion spectra of maltose hydroxyl-ethylated (hydroxyl-ethyl group could be attached to carbons 2 or 3 or 6 of the glucose units), mannito/sorbitol and sucrose obtained at two different collision energy (−10 and −35 eV) using the formiate adduct (*m/z* 227 for mannitol/sorbitol, *m/z* 431 for maltose hydroxyethylated and *m/z* 387 for sucrose) as parent mass.

Chromatographic conditions

In the literature, most applications using anion exchange chromatography were reported for the analysis of saccharides,[7,30–32] whereas for the analysis of polysaccharides, size exclusion chromatography (SEC) was suggested by the columns manufactures and utilized by previous investigators.[14] In the present study, amino stationary phase was used for the chromatographic separation of glucose, isomaltose, maltose, maltose hydroxyl-ethylated, mannitol, sorbitol and sucrose in human urine. The influence of mobile phase composition (percentage of the acetonitrile, type and amount of mobile phase modifier and ionic strength) and column temperatures (20, 40, 50, 60, and 80 °C) was evaluated in

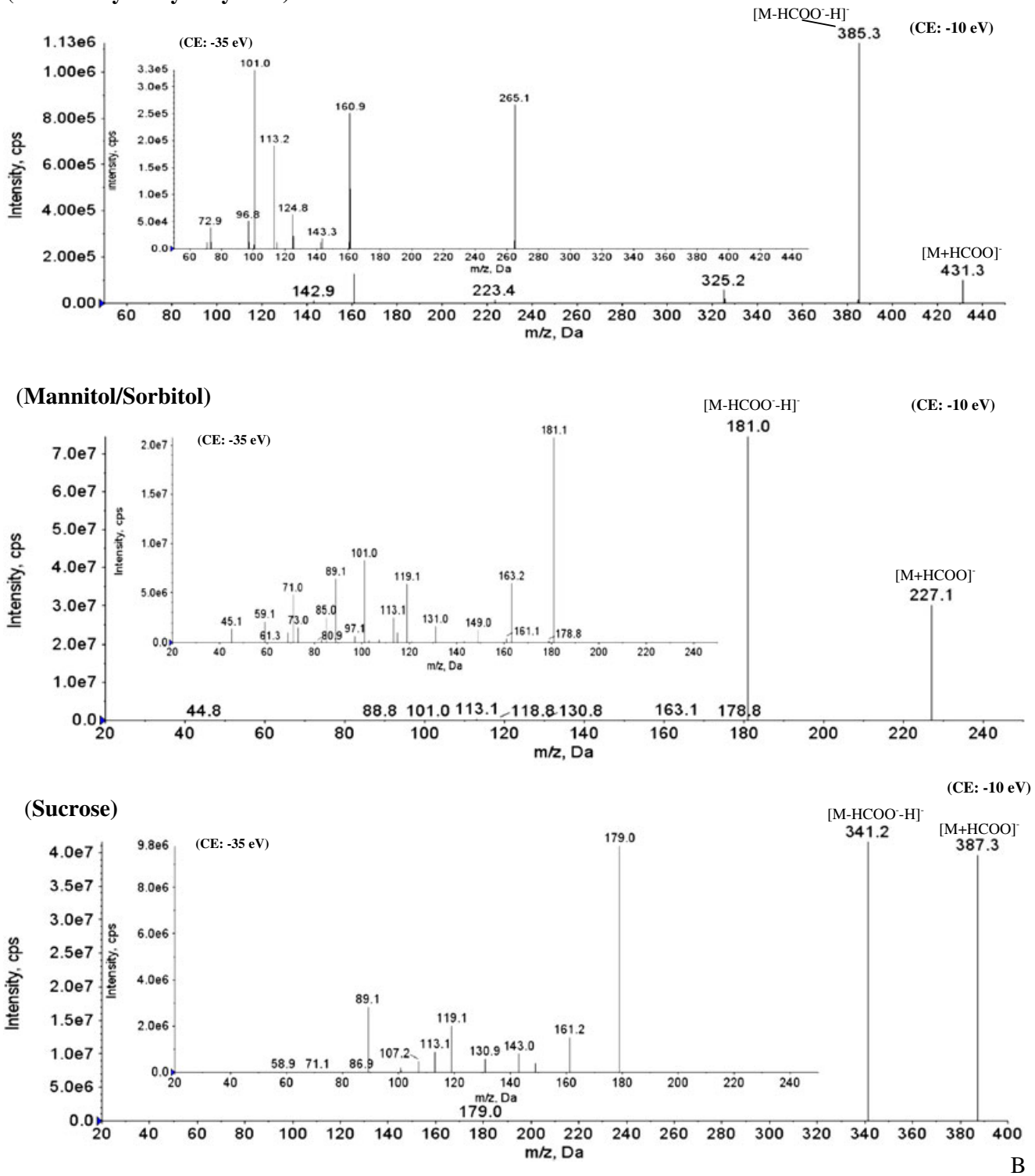

Figure 1. *(continued)*

order to obtain the best peak shape, chromatographic retention, sensitivity and selectivity.

Data collected showed that the highest chromatographic retention and selectivity were obtained using high percentage of acetonitrile (70–80%) at t = 0 and formic acid or acetic acid (pH 3.5–4.5) as mobile phase modifier. Whereas the best peak shape was obtained adding to the mobile phase a salt such as the ammonium formate or acetate. The salt concentration used was 5 mM because increasing the mobile phase ionic strength moderate differences in the analytes chromatographic retention, selectivity and peak resolution were observed, but at a salt concentration higher than 10 mM a decrease in sensitivity was measured.

Concerning the effect of the column temperature, data collected in this study are in agreement with previous researches,[33] showing that the transformation rate between the two anomers (α and β) of glucose, isomaltose, maltose hydroxyl-ethylated and maltose is temperature dependent. Specifically, while the elevated temperatures (higher than 60 °C) precluded the existence of specific isomers of carbohydrate, at a column temperature lower than 60 °C the two anomers, α and β, were separated.

The effectiveness of the optimized chromatographic conditions was tested analyzing a hydrolyzed standard solution of HES at a concentration of 1000 μg/ml and a standard mixture of glucose, isomaltose, mannitol, sorbitol and sucrose at a concentration of 300 μg/ml. In Figure 2, we can notice that an efficient separation of all compounds with the same molecular weight, apart from mannitol and sorbitol, was obtained using a column temperature of 40 °C, 0.1% of formic acid and 5 mM of ammonium formate as mobile phase additives and an initial concentration of acetonitrile of 80%.

Finally, the analysis of the hydrolyzed HES standard solution resulted in the detection of more than one peak at the retention time of maltose hydroxyl-ethylated with similar product ion spectrum (see the extract ion chromatogram reported in Figure 2); probably it was due to the presence of hydroxyethyl groups attached to carbons 2 or 3 or 6 of the glucose units. The identity of the structural isomers could not be determined due to the lack of individual reference compounds.

Assay validation

The optimized instrumental conditions were used to set up a screening procedure to detect the abuse of dextran, hydroxyethyl starch and mannitol in urine samples collected for doping control purpose, that was validated according to ISO 17025[24] and WADA guidelines.[25,26] For this purpose, ten aliquots of an artificial urine fortified with glucose, isomaltose, maltose, mannitol, and sucrose were analyzed and the linearity, accuracy, precision, LLOD, LLOQ,

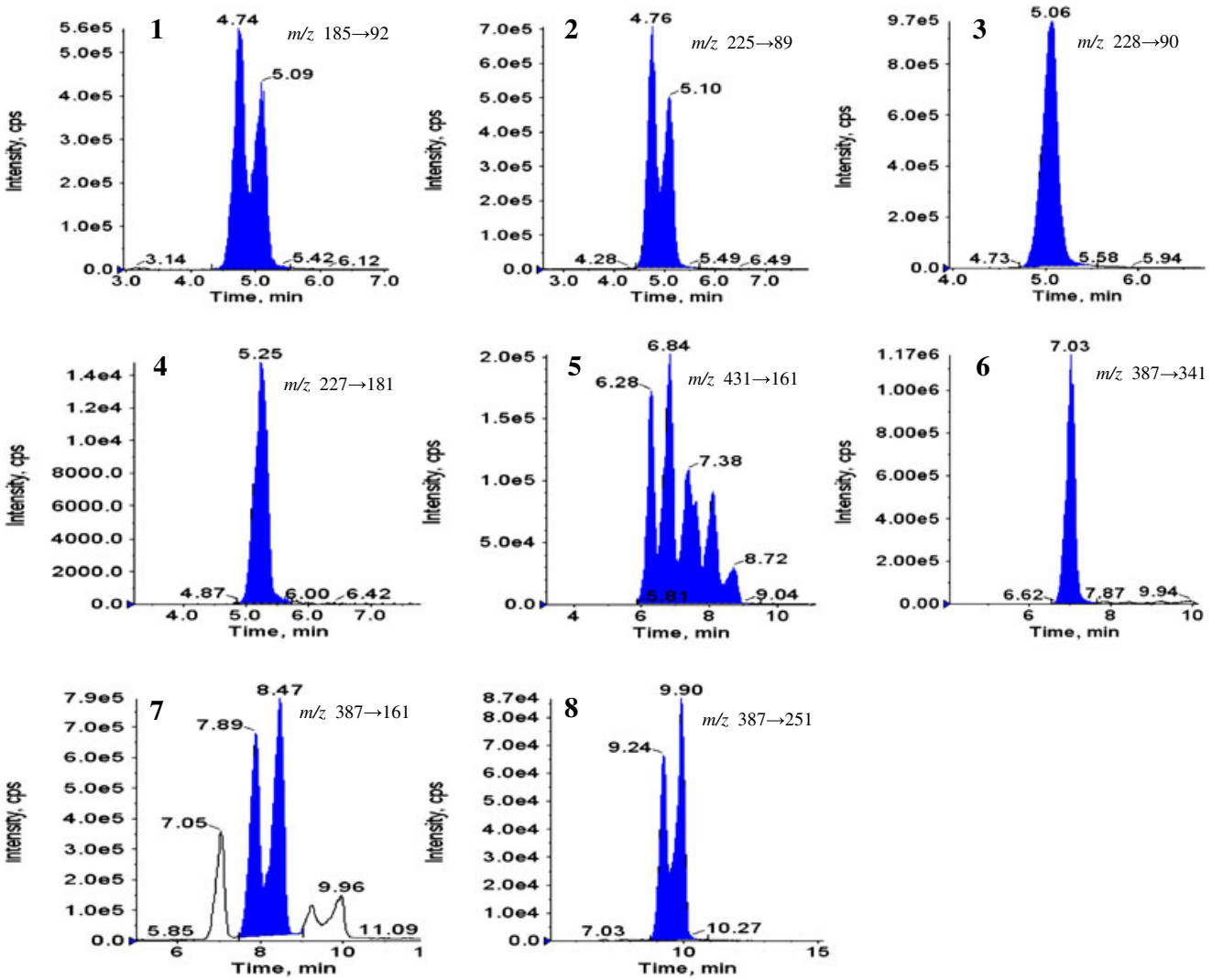

Figure 2. Extract ion chromatograms (XIC) of an LC-MS/MS analysis of a standard mixture of hydroxyethyl starch (1000 μg/ml), glucose (300 μg/ml), glucose-$^{13}C_6$ (300 μg/ml), isomaltose (300 μg/ml), mannitol (300 μg/ml), mannitol-$^{13}C_1$ (300 μg/ml) and sucrose (300 μg/ml) after enzymatic hydrolysis using the hydrolysis and instrumental conditions reported in the experimental section and in Table 1. Peak 1. α,β-Glucose-$^{13}C_6$ (ISTD1); Peak 2. α,β-Glucose; Peak 3. Mannitol-$^{13}C_1$ (ISTD2); Peak 4. Mannitol/Sorbitol; Peak 5. α,β-Maltose hydroxyl-ethylated; Peak 6. Sucrose; Peak 7. α,β-Maltose; Peak 8. α,β-Isomaltose. More than one peak at the retention time of maltose hydroxyl-ethylated was detected, due to the presence of hydroxyethyl groups attached to carbons 2 or 3 or 6 of the glucose units.

robustness were evaluated. Whereas for the determination of specificity, ion suppression/enhancement and repeatability of retention time and of relative abundances of selected ion transitions at least 20 blank urines were used.

Good linearity (R^2 0.990–0.995), reproducibility of the relative retention times (CV% lower than 1) and of relative abundances of selected ion transitions (CV% lower than 10) were measured for all compounds considered in this study. Carryover signal was not detected in blank samples injected just after the analysis of the fortified artificial urine at five times the LLOD concentrations. A suppression of ESI responses lower than 30% was observed at the retention times of the target analytes and internal standards while 20 different urine samples were injected. Concerning the specificity, being most of the analytes considered normally present in human urine, the identity of the target compounds was characterized by liquid chromatographic retention times and by the product ion spectra in 20 urines: no other compounds interfered with the target analytes or the internal standards. The LLOD and LLOQ were in the range of 30–100 μg/ml (Table 1). The deviation of the mean measured concentration from the theoretical concentration, for all compounds considered in this study, were considered acceptable, being below 15% for all the three levels tested (low, 200 μg/ml; medium, 500 μg/ml; high, 1000 μg/ml) using ten replicate each. Finally a good precision of peak areas (RSD) was obtained, not exceeding 10% for intra-day assays and 15% for inter-day assays for all analytes at low (200 μg/ml), medium (500 μg/ml) and high (1000 μg/ml) concentration level using ten replicates each.

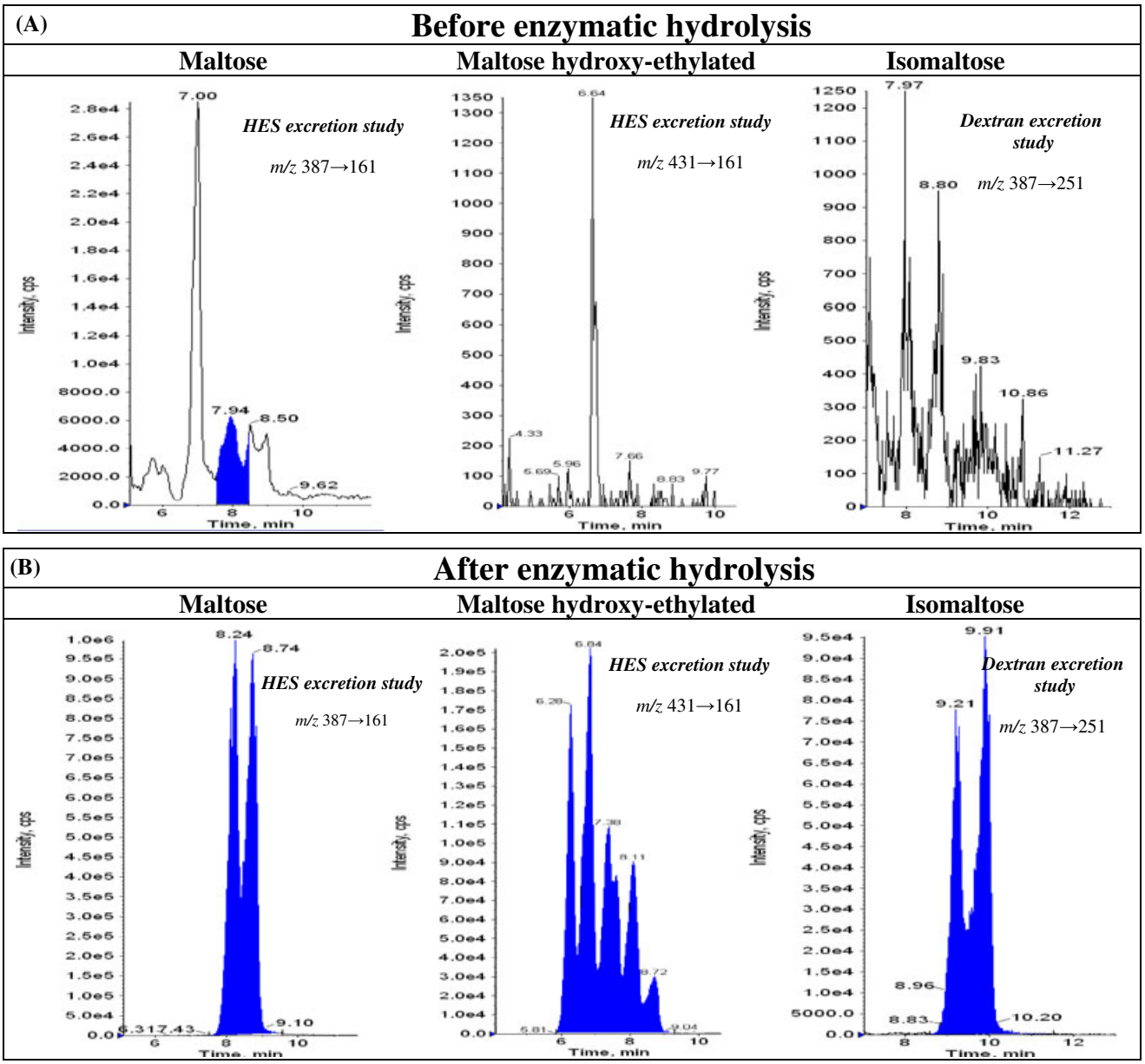

Figure 3. Extract ion chromatograms of maltose (*m/z* 387/161), maltose hydroxyethylated (*m/z* 431/161) and isomaltose (*m/z* 387/251) of an LC-MS/MS analysis of real samples obtained from excretion studies of dextran or hydroxyethylstarch-based pharmaceutical formulation before **(A)** and after **(B)** enzymatic hydrolysis, using the instrumental and the hydrolysis conditions reported in the experimental section and in Table 1. No peaks were detected at the retention times of maltose hydroxyl-ethylated and isomaltose before the enzymatic hydrolysis, on the contrary a peak at the retention time of maltose was detected also before the hydrolysis step.

Enzymatic hydrolysis conditions

Enzyme from different sources (α-amylase from human saliva and from *Bacillus sp.*; dextranase from *Penicillium sp.*), different enzyme concentrations (0.5, 1, 1.5, and 2 mg/ml) and different hydrolysis pH values (5, 6, 7), temperatures (20, 37, and 50 °C) and incubation times (10, 30, 60, and 120 min) were tested in order to obtained the best hydrolysis recovery.

The highest hydrolysis recovery was obtained using the α-amylase enzyme from human saliva at a concentration of 1 mg/ml (10 µl) for hydroxyethyl starch and the enzyme dextranase from *Penicillium sp.* at a concentration of 1.5 mg/ml (10 µl) for dextran,

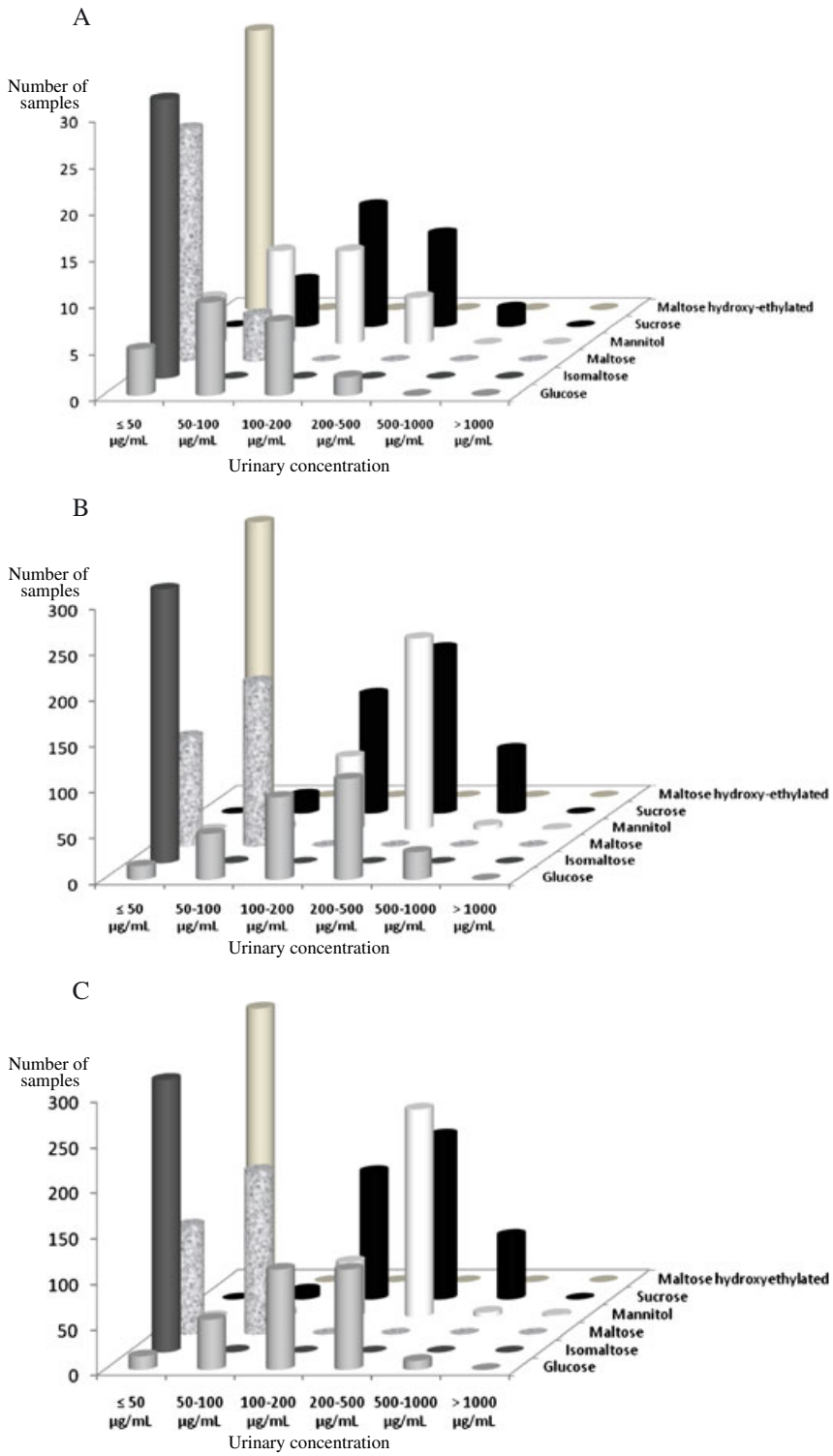

Figure 4. Saccharides (glucose, isomaltose, maltose hydroxyl-ethylated, maltose and sucrose) and mannitol physiological ranges in 30 urine samples from healthy volunteers not using any medication **(A)**, in 300 routine samples collected in competition **(B)** and in 300 routine samples collected out of competition **(C)**. Higher mannitol urinary concentration levels were measured in the regular doping control urines, without significant differences between IC and OOC.

the phosphate buffer (pH 6), a temperature of 37 °C and an incubation time of 60 min.

The lowest level detectable in urine sample was 100 μg/ml for dextran and higher than 200 μg/ml (the limit of detection of the GC-MS method currently used by the accredited anti-doping laboratory of Rome[13]) for HES.

The effectiveness of the developed hydrolysis procedure for doping control purpose (where a complete hydrolysis is not usually necessary to discriminate a negative from a positive sample) was tested analyzing urine samples obtained from excretion studies of dextran or HES-based pharmaceutical formulations. Specifically, Figures 3A and 3B show the extract chromatograms of a urine samples collected after the administration of dextran or HES-based pharmaceutical formulation before (Figures 3A) and after (Figures 3B) enzymatic hydrolysis with dextranase from *Penicillium sp*. for dextran and with α-amylase from human saliva for HES. We can notice in both cases that before the enzymatic hydrolysis no peaks were detected at the retention times of the selected hydrolysis products of HES and dextran, maltose hydroxyl-ethylated and isomaltose respectively (see extracted ion chromatograms and product ion spectra reported in Figure 3A). On the contrary, after the hydrolysis with α-amylase from human saliva and dextranase from *Penicillium sp*. peaks at the retention time of maltose hydroxyl-ethylated for the sample collected after the administration of HES-based pharmaceutical formulation and at the retention time of isomaltose in case of the sample collected after the administration of dextran-based pharmaceutical formulation were detected (see extracted ion chromatograms and product ion spectra reported in Figure 3B). Maltose was not selected as marker of HES abuse because, as shown in Figure 3A, it is normally present in urine.

Urinary concentration values of saccharides and polyalcohol in a reference population

Due to the fact that most of the analytes considered in this study are normally present in fruit and are widely used as food and drugs additives, the newly developed method was used to establish their physiological urinary concentration ranges. For this purpose, a total of 600 regular doping control urines tested negative in all screening procedures and collected in competition (IC, 300 samples) as well as out of competition (OOC, 300 samples) testing of different sports disciplines (cycling, basketball, football, swimming, rugby, and volleyball) and 30 urine specimens from healthy volunteers were analyzed for the presence of glucose, isomaltose, maltose, maltose hydroxyl-ethylated, mannitol, and sucrose in order to establish the physiological urinary concentration ranges. Data collected showed that isomaltose and maltose hydroxyl-ethylated, the hydrolysis products selected to detect the abuse of dextran and hydroxyethyl starch respectively, were not present in all urines (Figures 4A–4C). On the contrary the urinary maltose (another hydrolysis product of HES) concentration was in the range of 30–100 μg/ml, the urinary glucose concentration was in the range of 30–500 μg/ml, the urinary mannitol concentration was in the range of 30–500 μg/ml and the urinary sucrose concentration was in the range of 70–1000 μg/ml for the urines collected from laboratory staff (Figure 4A); whereas for the doping control samples in the range of 30–100 μg/ml for maltose, in the range of 60–1000 μg/ml for glucose, of 100–1200 μg/ml for mannitol and of 100–1000 μg/ml for sucrose (Figures 4B and 4C). Our results are in agreement with previous research[7] and confirm the necessity to establish criteria of positivity for mannitol. Specifically as shown in Table 2, in five (0.8%) samples mannitol levels were below the LLOQ of 70 μg/ml, 15 (2.4%) urines contained mannitol between 50 μg/ml and 100 μg/ml, 150 (24%) urines between 100 μg/ml and 200 μg/ml, 447 (71%) samples between 200 μg/ml and 500 μg/ml, 10 (1.6%) urines between 500 μg/ml and 1000 μg/ml, and 3 (0.5%) samples higher than 1000 μg/ml. The maximum mannitol concentration measured was 1200 μg/ml, at least 20 times lower than the reported[7] concentration reached after intravenous administration. Furthermore mannitol urinary levels higher than 200 μg/ml were found more often in the regular doping control urines, without differences between IC and OOC (Table 2), due to a potential oral administration of drugs/nutritional supplements containing mannitol as additive. The mannitol physiological urinary levels were not influenced by the co-elution with sorbitol because the sorbitol physiological concentration, as reported by previous investigators,[7] is lower than the limit of quantification (70 μg/ml) of the newly developed screening procedure.

Table 2. Urinary mannitol concentration found in 600 doping control urine samples derived from in competition as well as out of competition testing of different sports disciplines and in 30 urine samples from laboratory staff not using any medication

	Samples			
Urinary concentration (μg/ml)	**IC (300 samples)**	**OOC (300 samples)**	**Reference population (30 samples)**	**Total**
≤ 50	0.0%	0.0%	17.0%	5
50-100	2.0%	2.0%	33.0%	15
100-200	27.0%	20.0%	33.0%	150
200-500	70.0%	76.0%	17.0%	447
500-1000	0.7%	1.4%	0.0%	10
≥ 1000	0.3%	0.6%	0.0%	3

Conclusions

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 the abuse of dextran, hydroxyethyl starch, and mannitol in doping control. The sample pretreatment procedure and the instrumental run-time is rather short compared to the GC-MS or LC-MS/MS procedures currently used for the screening and confirmation analysis of mannitol, dextran, and HES. On the contrary, compared to the recently proposed strategies[14,15] to screen in a very short analysis time dextran and HES using in-source collision-induced dissociation, the analytical procedure here presented is longer due to the enzymatic hydrolysis step before the instrumental analysis. Nevertheless, the newly developed method, 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 isomaltose (the hydrolysis products selected as markers of HES and dextran abuse respectively; Figures 1A and 1B), could be used also as HES and dextran confirmation method avoiding the need for the complex confirmation procedures used until now. Whereas for mannitol confirmation, due to the co-elution with sorbitol, established method is available that enables its unambiguous identification.[7] Furthermore, as shown by the investigation of the saccharides and polyalcohols, physiological urinary ranges in

630 urine samples a threshold value for mannitol might be established in order to enable doping control laboratories to report abnormal analytical findings. The number of urine samples examined in this study is not sufficient for a statistical evaluation and consequently to fix a mannitol threshold, thus further studies should be conducted to confirm our results and to establish criteria of positivity. Finally, HES, dextran, and mannitol administration studies (oral and intravenous) should be conducted to establish a way to discriminate between the permitted oral and the prohibited intravenous administration.

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