Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (11)

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Sport und Buch Strauß, Köln, 2003

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (11). Sport und Buch Strauß, Köln, (2003) 119-128

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MALDI TOF mass spectrometry screening for the presence of plasma volume expanders in urine

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ABSTRACT

The use of plasma volume expanders, especially those based on chemically modified polysaccharides such as hydroxy ethyl starch (HES), has found its way from the medical field to the athletic community in the everlasting drive for performance enhancement. The placement on the list of banned substances by the International Olympic Committee, in turn requires accurate and sensitive analytical tools for its detection in complex biological matrices. Here we present an relatively straight forward method for the detection of HES in urine, based on the carefully controlled partial acid hydrolysis of urine (20 µl) in a total volume of 500 µl. Following the incubation (30 minutes) an aliquot of the hydrolysate is dried, re-suspended in the analytical matrix and examined by MALDI TOF mass spectrometry. The resulting MS profiles reveal a high number of characteristic peaks in the mass range between 500 and 3000 Da, a region that in urine samples devoid of HES appears relatively clean, and thus allows its unambiguous identification. This approach is fast (the mass profile can be obtained within 90 minutes), highly sensitive (the effective sample amount on the MALDI target is equivalent to 100 nl urine), requires little sample handling (4 steps), requires no derivatisation and is devoid of interference from other biomolecules. In addition, the approach can be applied to other polymer derived plasma expanders such as dextran and acetyl starch.

INTRODUCTION

Plasma volume expanders that have been developed as a plasma substitute in the treatment of haemorrhage[1] or hypovolaemic shock[2], and for cryo-protection of biological material[3] include protein structures such as albumin and gelatin, naturally occurring carbohydrate polymers such as dextran, and chemically modified polysaccharides such as hydroxyethylated starch (HES). Especially the latter, investigated since 1957, has found widespread acceptance because of the limited adverse reactions[4] and the fact that the half-life time can be carefully controlled based on the average degree of molar substitution[5].

Athletes, often through their medical consultants, have rapidly learned that this substance on its own can be used to prevent dehydration and thus enhance endurance[6,7]. On its own, or in combination with recombinant erythropoietin its use may result advantageous as an elevated blood volume and haemoglobin are achieved while hematocrit levels remain within

the legal range. Elevated hematocrit levels may be indicators of possible erythropoietin abuse and may result in a non-start ruling and trigger urine testing. This has resulted in the inclusion of HES, as well as other plasma volume expanders, on the list of prohibited substances by the International Olympic Committee (IOC) since January 2000. As a consequence two analytical methods, based on gas chromatography and mass spectrometry, have been developed for the analysis of HES[8,9]. These methods are compatible with complex matrices such as urine but are relatively laborious, time consuming, and include one or more derivatisation procedures. In addition, generated monosaccharides may partially co-elute in the chromatogram with other, glycoprotein derived, monosaccharides that share a number of so-called identifier ions (*e.g.* m/z 191, 204 and 217) giving rise to altered ion-intensity ratios and occasionally uncertain doping-control results. Here we present an alternative, relatively straight forward method for the detection of HES in urine, based on the carefully controlled partial acid hydrolysis and direct analysis by MALDI TOF mass spectrometry following evaporation to dryness. This approach complies with the requirements for anti-doping control in terms of speed, sensitivity, ease, etc. and does not suffer from the drawbacks of the existing analytical tools. In addition, the scope of the approach is broader as it will detect the presence of other polymer derived plasma expanders such as dextran and acetyl starch[10,11].

EXPERIMENTAL

Materials - Hydroxyethyl starch (HETASTARCH; 6% solution in 0.9% sodium chloride) with an approximate molecular weight of 450 kDa and a molar degree of substitution 0.7 was purchased from Sigma Chemical Co (Madrid, Spain). 2,5-Dihydroxy benzoic acid (DHB), 2-hydroxy-5-methoxy benzoic acid, α-cyano-4-hydroxy cinnamic acid, and ferrulic acid were purchased from Sigma Chemical Co (Madrid, Spain). Dextran T70 was purchased from Pharmacia Biotech (Uppsala, Sweden). All other reagents employed were of the highest purity commercially available.

Biological samples - Different specimens were used for the development and evaluation of the protocol. Blank urines collected from volunteers and urine samples from professional athletes participating in endurance sports were used as negative controls. Positive control included urine samples from individuals participating in excretion studies in Köln (Germany) and Oslo (Norway) as well as from hospitalised patients receiving ELOHES® (Fresenius-Laboratorios Mein SA, Spain) treatment; samples were collected between 12 and 24 hours after the intravenous administration of 200-500 ml 0.9% (w/v) sodium chloride containing 6% (w/v) hydroxyethyl starch. All samples were stored at 4°C prior to analysis.

Monosaccharide analysis - Samples were analysed essentially as described by Thevis *et al.* [12] with the following modification: In stead of 3M hydrochloric acid (HCl), 4M trifluoro acetic acid (TFA) was used.

Partial acid hydrolysis - To a screw-capped test tube containing 0.5 ml of 4M TFA, 20 μl sample solution were added and mixed thoroughly. Sample solutions consisted of aqueous HES or dextran solution (2 μl and 18 μl deionised water), blank urine (20 μl), spiked urine (2 μl HES or dextran and 18 μl blank urine), urine from athletes or excretion studies (20 μl). Solutions were incubated for 15, 30, 45 or 60 min at 100°C in a aluminium heating block. Subsequently, samples were cooled on ice, an aliquot (50 μl) was transferred to a conical glass microvial insert and immediately dried by rotary evaporation under reduced pressure.

Sample preparation for MALDI analysis - In general matrices were prepared by dissolving approximately 10 mg in 1 ml deionised water-methanol (1:1, v/v) in an Eppendorf vial. Prior to use matrix solutions were centrifuged at 11.000 rpm for 5 min. The dried samples were resuspended in 10 μl matrix solution and an aliquot (100-150 nl) deposited on the target plate. Samples were allowed to crystallise at room temperature using different approaches (*e.g.* air drying, vacuum drying, recrystallisation from MeOH, *etc.*) [13, 14].

Instrumentation

GC-MS – The qualitative analysis was carried out by GC on a capillary ULTRA 2 column (12 m * 0.33 mm, i.d. 0.2 mm, composition: (5%)-diphenyl-(95%)-dimethylsiloxane, J&W Scientific) using a Hewlett Packard 5890 (series II) gas chromatograph (temperature program 140–240 °C at 8 °C/min) and a Hewlett Packard 5971A quadrupole mass spectrometric detector. The identification of the sample constituents was performed according to Kamerling and Vliegenthart[15].

Positive-ion mode MALDI TOF analyses were performed on a Voyager-DE™ STR Biospectrometry workstation (Applied Biosystems, Framingham) operating at an accelerating voltage of 19 kV (grid voltage 67%, ion-guide wire 0.05%) and equipped with a N_2 laser (337 nm). Delay times were varied between 100 and 200 ns and the reflector mirror voltage ratio set to 1.12. Typically, 100 mass scans were recorded in the mass range 500 – 3500 Da. Mass spectra were externally calibrated using well defined glucose oligomers. Recorded data were processed using Data Explorer™ Software (Applied Biosystems, Framingham).

RESULTS AND DISCUSSION

Exploration of optimum hydrolysis conditions - Acid hydrolysis of the different polysaccharides was performed using a variety of experimental setting in order to assess optimal conditions for rapid screening. Different acidic solutions (hydrochloric acid, acetic acid, trifluoro acetic acid, sulphuric acid) resulted in similar profiles when equimolar (1, 2, 3, and 4 M) conditions were employed (data not shown).

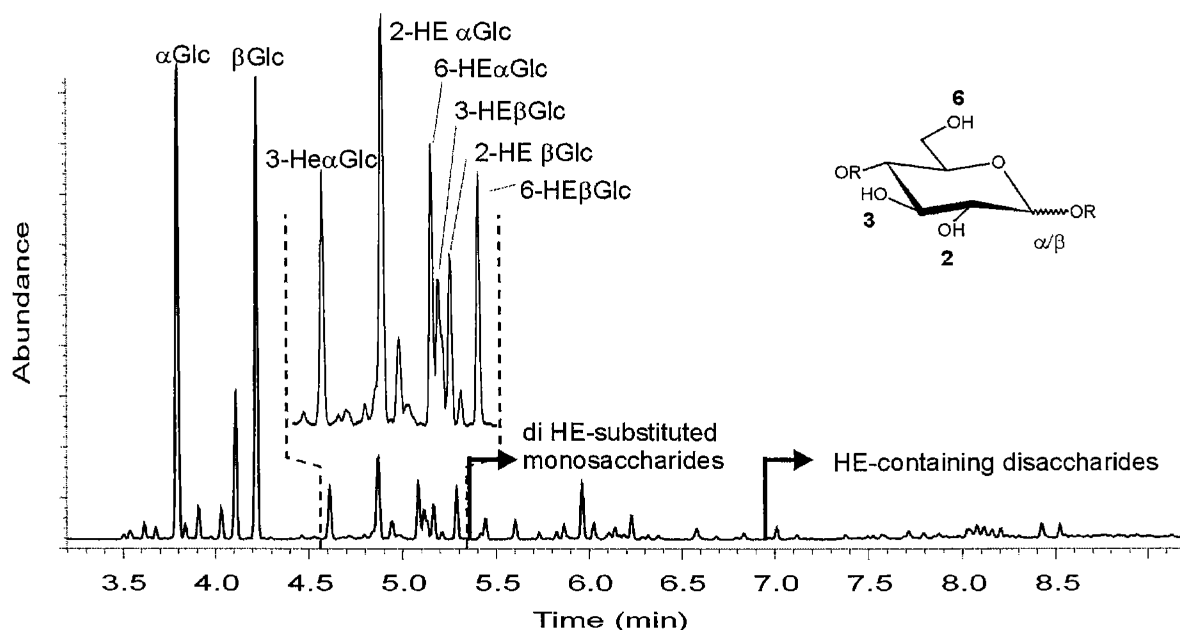

Figure 1. Total-ion current gas chromatogram of hydroxyethyl starch after hydrolysis for 90 min with 4M trifluoro acetic acid at 100 °C. The region of substituted mono-hydroxyethyl monosaccharides is highlighted. Also indicated are the region where multiple hydroxyethyl substituents containing monosaccharides and disaccharides elute.

Trifluoro acetic acid (4M) was selected because of the nearly complete hydrolysis within 90 min (confirmed by monosaccharide analysis, Figure 1[8]) and the volatile nature of the acid which allows rapid sample work-up. Partial acid hydrolysis of the polysaccharides was explored as a function of incubation time while maintaining the temperature constant (100 °C). With increasing time the mass-envelopes shifted towards lower mass values and dropped below 2000 Da for times longer than 45 min. A 30-min incubation resulted in a desired molecular weight distribution in the mass range between 500 and 3500 Da (Figure 2) for dextran 70 and in the mass range between 500 and 2800 Da (Figure 2) for hydroxy ethyl starch. Repetitive experiments gave similar results although with slight fluctuations in the absolute mass range due to the dynamic nature of the hydrolysis. With these conditions it was anticipated to observe lower molecular weight distributions for biological samples as these polymers will only be secreted into urine after partial degradation in the liver [5,16,17,18].

Evaluation of the mass profiles - The mass spectra for dextran 70 resulted in relatively simple mass spectra with sodiated pseudo-molecular ions containing 18 original peaks with a 162.053 Da mass spacing (Figure 2, Table I). In addition, every sodiated pseudo-molecular ion was accompanied by a low-intensity dehydrated species.The mass spectrum of the partially hydrolysed hydroxy ethyl starch displayed a more complex profile with a large number of peaks (over 160) in the mass range of 500 – 2500 Da (Figure 3).

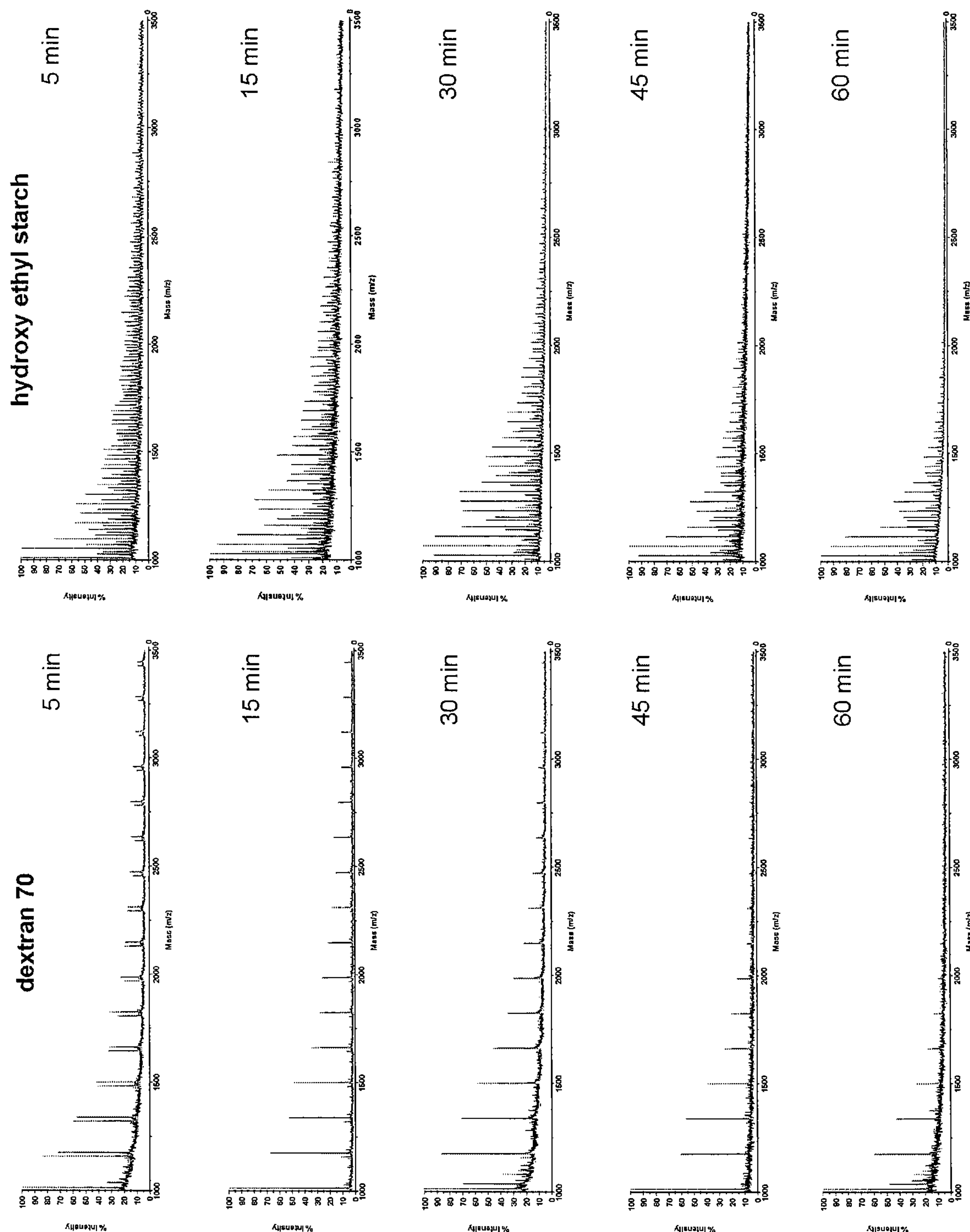

Figure 2. MALDI TOF mass spectra of both dextran 70 (series on the left-hand side) and hydroxyethyl starch (series on the right-hand side) after partial acid hydrolysis with 4M TFA at 100 °C.

Direct peak-matching against theoretical masses, taking into account the fact that above ~ 2200 Da the (M+1)-isotope predominates for carbohydrates, resulted in the identification of 87 sodiated pseudo-molecular ions covering all high-intensity signals in the spectrum (Table I). In addition several low-intensity peaks (7 to 13 % of the base peak) could be addressed as potasiated pseudo molecular ions. A total of 35, medium to low intensity (10 to 20% of the base peak) could be assigned to $[M - H_2O + X]^+$ (X, Na or K). Only a limited number of

signals (<5%) could not readily be attributed to a particular structure. Altogether, partial acid hydrolysis of both dextran and hydroxy ethyl starch yield unique mass profiles that can be used for identification.

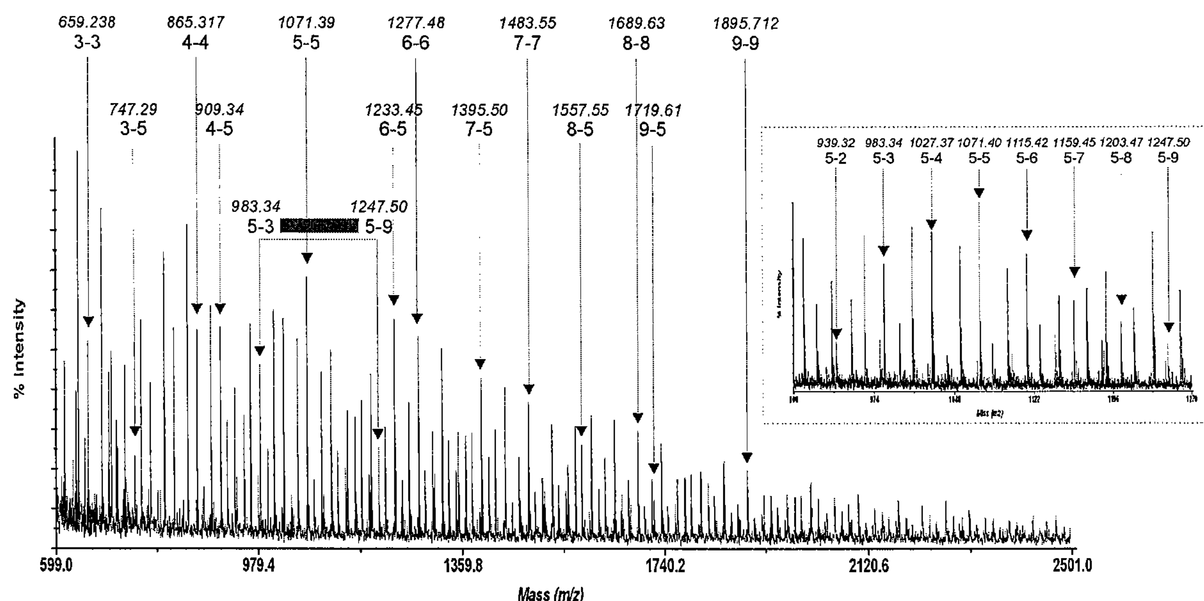

Figure 3. MALDI-TOF mass spectrum of hydroxy ethyl starch after partial acid hydrolysis. Assignments are indicated in the figure, e.g. 3-5 corresponds to a trisaccharide bearing 5 hydroxy ethyl substituents ($[C_{28}H_{52}O_{21}]^{Na+}$; 747.2911 Da). Indicated are mono-substituted oligosaccharides (3-3 till 9-9), penta-substituted oligosaccharides (3-5 till 9-5) and differently substitute pentasaccharides (5-2 till 5-9, amplification).

Interestingly, the average molar substitution (MS) in hydroxy ethyl starch, derived from the fragments analysed is very close to 1.0 with 36 mass values showing a MS value above 1 and an equal number of peaks displaying a MS value below 1 (Table II). The observed average MS value is thus higher than the value indicated by the manufacturer (0.7) and unlikely to be originated by preferential cleavage of glycosidic bonds of substituted residues. In view of the importance of the average MS of plasma volume expanders for controlling the half-life time [5] due to the resistance to enzymatic degradation[17] the approach described here would facilitate more accurate data than those obtained with current methods to characterise polymeric plasma volume expanders [19] and hence permit a more detailed knowledge of structure-function relationship of these polymers. Subsequently, biological samples including blank and spiked urine samples (with hydroxy ethyl starch), urine samples from elite athletes, specimen from participants in secretion studies and urine samples from patients receiving hydroxy ethyl starch treatment were analysed. In parallel to the MALDI TOF analyses all urine were analysed for the monosaccharide content. The chromatograms (data not shown) of blank specimens only revealed the presence of free α/β-glucose in minor amounts whereas hydroxy ethyl starch containing urine displayed the characteristic peaks belonging to free α/β-glucose, 2-hydroxyethyl-α/β-glucose, 3-hydroxyethyl-α/β-glucose, and 6-hydroxyethyl-α/β-glucose.

Mass spectra recorded in 2,5-DHB for blank urine samples, both before and after acid treatment (data not shown), yielded low intensity noise levels in the selected mass range.

Table I. Theoretical mono-isotopic mass distribution of, sodium-cationised, dextran (#HE 0) and hydroxy ethyl starch (#HE 1-13) derived oligosaccharides. On the Y-scale the number of monosaccharides is indicated and on the X-scale the number of hydroxy ethyl groups. Masses beyond the scope of the MALDI mass range and impossible variants have been left out. Grey-shaded cells indicate a molar distribution of 1.0. Mass values in bold were observed in the experimental data.

#	# HE 0	1	2	3	4	5	6	7	8	9	10	11	12	13
1	180,06	247,08	291,11	335,13										
2	342,12	409,13	453,16	497,18	541,21	585,24	629,26							
3	504,17	571,19	**615,21**	**659,24**	**703,26**	**747,29**	**791,32**	835,34	879,37	923,39				
4	666,22	**733,24**	**777,26**	**821,29**	**865,32**	**909,34**	**953,37**	**997,40**	**1041,42**	1085,45	1129,47	1173,50	1217,53	
5	828,27	**895,29**	**939,32**	**983,34**	**1027,37**	**1071,40**	**1115,42**	**1159,45**	**1203,47**	**1247,50**	1291,53	1335,55	1379,58	1423,61
6	990,33	1057,34	**1101,37**	**1145,40**	**1189,42**	**1233,45**	**1277,47**	**1321,50**	**1365,53**	**1409,55**	**1453,58**	1497,61	1541,63	1585,66
7	1152,38	1219,40	**1263,42**	**1307,45**	**1351,48**	**1395,50**	**1439,53**	**1483,55**	**1527,58**	**1571,61**	**1615,63**	**1659,66**	**1703,68**	**1747,71**
8	1314,43	1381,45	**1425,48**	**1469,50**	**1513,53**	**1557,55**	**1601,58**	**1645,61**	**1689,63**	**1733,66**	**1777,69**	**1821,71**	**1865,74**	**1909,76**
9	1476,49	1543,50	1587,53	1631,55	**1675,58**	**1719,61**	**1763,63**	**1807,66**	**1851,69**	**1895,71**	**1939,74**	**1983,76**	**2027,79**	**2071,82**
10	1638,54	1705,55	1749,58	1793,61	**1837,63**	**1881,66**	**1925,69**	**1969,71**	**2013,74**	**2057,76**	**2101,79**	**2145,82**	**2189,84**	**2233,87**
11	1800,59	1867,61	1911,63	1955,66	1999,69	**2043,71**	**2087,74**	**2131,77**	**2175,79**	**2219,82**	**2263,84**	**2307,87**	**2351,90**	**2395,92**
12	1962,64	2029,66	2073,69	2117,71	2161,74	2205,77	**2249,79**	**2293,82**	**2337,84**	**2381,87**	**2425,90**	**2469,92**		
13	2124,70	2191,71	2235,74	2279,77	2323,79	2367,82	2411,84	2455,87	2499,90					
14	2286,75	2353,77	2397,79	2441,82	2485,85									
15	2448,80													

The mass spectra from urine samples spiked with hydroxy ethyl starch before acid treatment (Figure 4A), and after partial acid hydrolysis (Figure 4B) clearly reflect the requirement of the acid hydrolysis in order to observe the characteristic profile. Mass spectra recorded for urine specimens from secretion studies before and after partial acid hydrolysis (Figure 5A and 5B, respectively) showed very similar characteristics to those obtained from spiked urines. Urine samples from elite athletes before and after acid treatment (data not shown) did not display the typical HES-mass profile, even though the glucose content proved beyond the value considered as normal. Finally, specimens taken from several hospitalised patients with complex medication, amongst which hydroxy ethyl starch, were analysed.

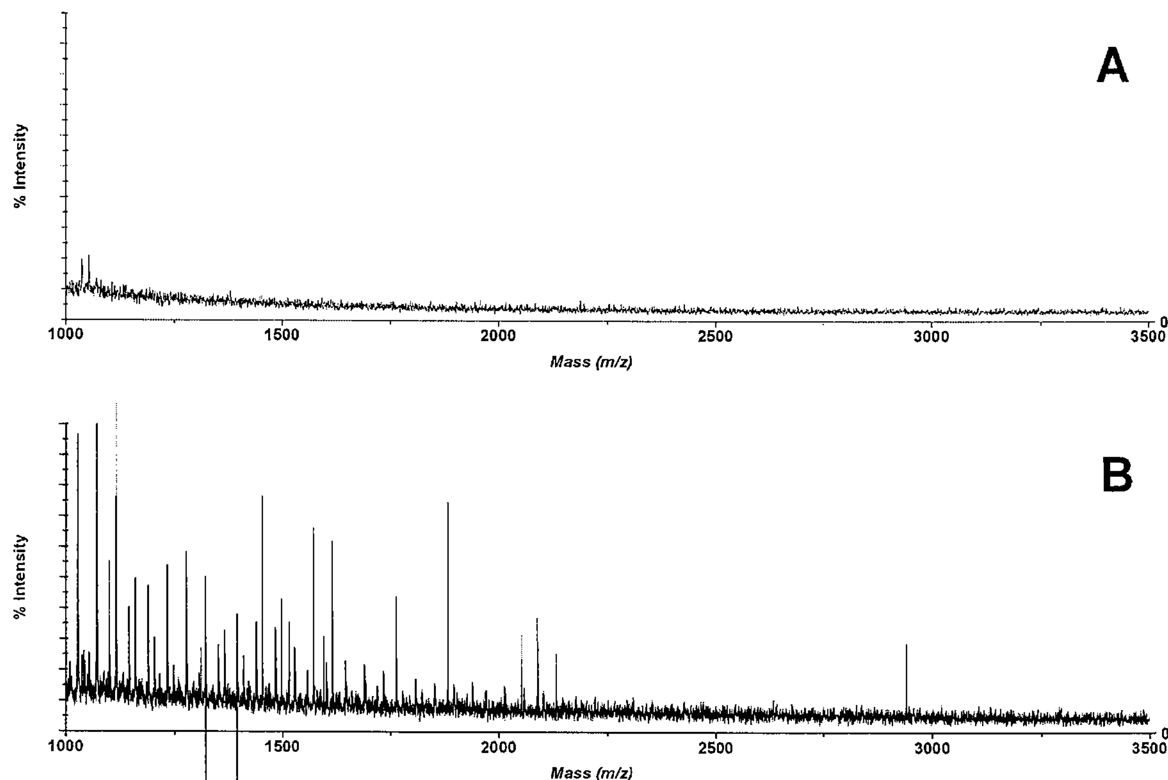

Figure 4. MALDI TOF mass spectra from A, blank urine sample used in standard doping analysis, spiked with hydroxy ethyl starch. B, blank urine sample spiked with hydroxy ethyl starch after partial acid hydrolysis.

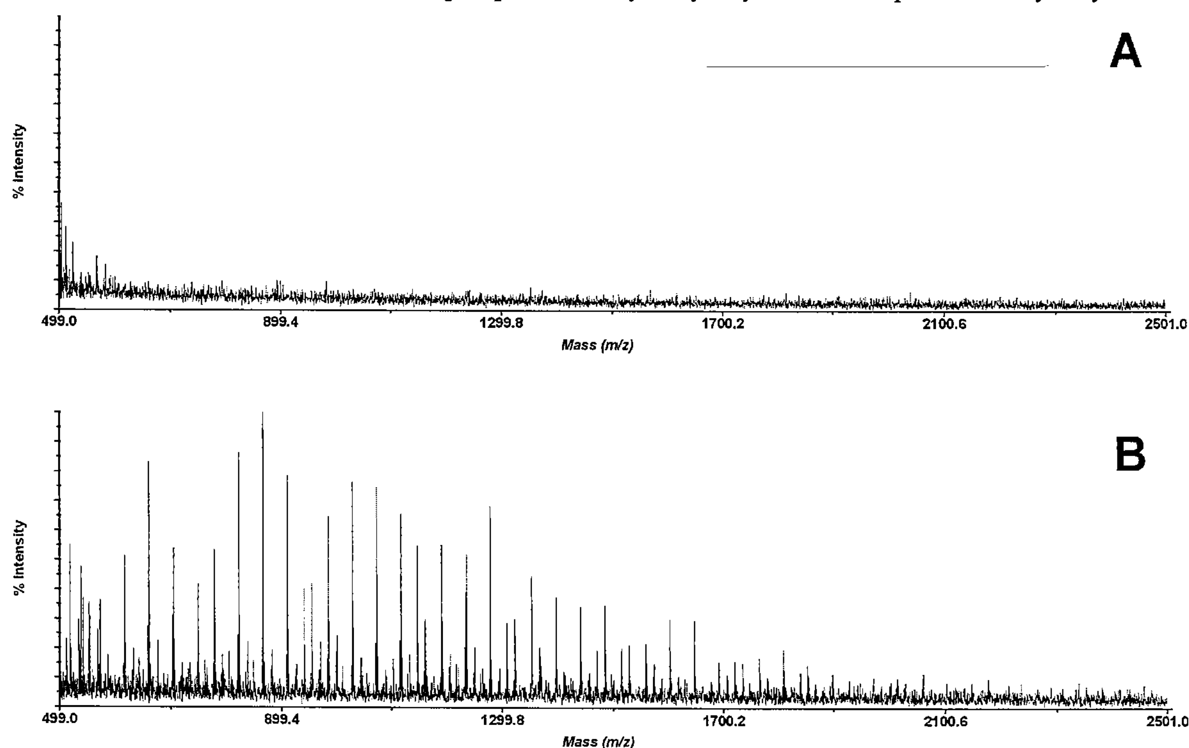

Figure 5. Representative MALDI TOF mass spectra from urine specimens obtained from secretion studies before (A) and after (B) partial acid hydrolysis (PAH). Samples were collected 12-24 hours post-administration of 500 ml 6% (w/v) hydroxy ethyl starch solution.

Samples were taken at random time-points after administration of different doses of hydroxy ethyl starch (ELOHES, 6% (w/v) in 0.9% NaCl). All specimens could be shown to contain hydroxy ethyl starch (data not shown) due to the large number of identifiers, yet mass spectra were not as neat as those observed for secretion-study specimens. Noteworthy is the observation that although the complex biological matrix results in an amorphous, transparent crystallisation which results difficult to ionise, the analyte appears to concentrate in microcrystalline regions that facilitates data acquisition. Different crystallisation procedures, such as air drying, vacuum drying, thin layer deposition, etc. were examined but none of these methods seemed to enhance significantly the formation of these microcrystalline areas. The addition of NH_4F to DHB[14] did not substantially meliorate the mass spectrum although slightly higher ion-intensities were observed in the higher mass region.

CONCLUSIONS

MALDI-TOF mass spectrometry analyses of biological samples after controlled acid hydrolysis permits the unambiguous identification of different polymeric plasma volume expanders such as dextran and hydroxyethyl starch. The sensitivity, cost-efficiency, speed, lack of extensive sample preparation, and coverage of distinct products that can be detected in a single analysis renders this simple approach highly attractive as a standard analytical tool for doping-control laboratories. Using the conditions as determined, a partial acid hydrolysis results in a characteristic mass profile between 500 and 2800 Da, a mass range considered ideal for the analysis of oligosaccharides by MALDI-TOF ms. Furthermore, in this mass range and using 2,5-DHB as matrix no interference from other compounds present in a complex biological matrix such as urine are observed either before or after acidic treatment thus minimising the risk of a so-called false-positive. This approach uses as little as 20 µl urine, from which eventually only 0.5% is deposited on the MALDI target, is complementary to the method published by Thevis *et al.*[8] in terms of identification of HES. However, it offers the advantage of reduced sample preparation and the absence of a derivatisation step. Furthermore, the approach covers the actually used polysaccharide-based plasma volume expanders and will be able to pick-up the illicit use of newly developed products such as acetylated starch[10,11] without additional effort. A careful study of the data obtained for the hydroxyethyl starch oligosaccharides revealed an average molar substitution of 1.0 whereas product specifications indicated a lower (0.7) value. Such precise determination, evidently not covered by the actual routine quality control, is of utmost importance for pharmaceutical enterprises, as these products are intended for medical purposes and different properties have been attributed as a function of this parameter.

Acknowledgments

The authors gratefully acknowledge Prof. Dr. W. Schänzer (German Sports University Cologne, Cologne, Germany) and Prof. Dr. P. Hemmersbach (Norwegian University of Sport

and Physical Education, Oslo, Norway) for providing us with urine samples from secretion studies. We also thankful for the urine samples from hospitalised patients (Hospital del Mar, Barcelona, Spain) kindly provided by Dr. S. Abanades. This study was supported by the Generalitat de Catalunya (Grant n° 2001SGR00407).

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