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GC/MS-Detection of Hydroxyethyl Starch (HES) in Human Urine

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Introduction

HES is not a prohibited substance according to the IOC doping-list[1] and is usually administered in cases of hypovolaemic shocks[2], disturbances in the capillary blood circulation and as a cryoprotectant, but recent data showed that it is definitely used in sports may be to control the haematocrit value, to raise the amount of body fluid before endurance sports or to protect frozen red blood cells from freezing damage[3]. Several investigations dealing with the importance of hydration in sports state that dehydration causes a decreased plasma volume and an impaired thermoregulation being responsible for a serious loss of exercise performance.[4 5] Additionally the possible HES application by autologous transfusion of frozen stored blood should be taken into consideration.

The good physiological results corresponding to metabolism, immunogenicity, adverse reactions and half-life in human bodies achieved by the use of HES are reasons for its preference as a plasma volume expanding substance.

HES has a close chemical similarity to glycogen, so that it lacks immunogenicity but the small differences resulting from alkylation of hydroxy functions with hydroxyethyl groups decrease the speed of clearance by α-amylase cleavage in the blood system enormously[6]. In fact it is possible to control the half-life of HES in the human body by its degree of substitution.

The heterogeneity of HES is the reason for its complex and dynamic metabolism in which new but smaller HES molecules are formed and distributed into organs or excreted into urine and bile[7] . But studies showed only a recovery of less than 1% of the administered dose in feces over a 14 day period.[8]

The basic structure of HES is a polymer of 1,4-linked α-D-glucose with a branching degree of about 6% at the oxygen located at C6. It is usually derived from corn or sorghum amylopectin by reaction with ethylene oxide in the presence of an alkaline catalyst[9]. The derivatisation of hydroxy groups is possible at the positions 2, 3 and 6 and there is a distribution of 70% 2-, 20% 6- and 10% 3-substitution due to the reactivity of the hydroxy groups. The total degree of substitution is different in the remedies sold and varies from 35 up to 70%.

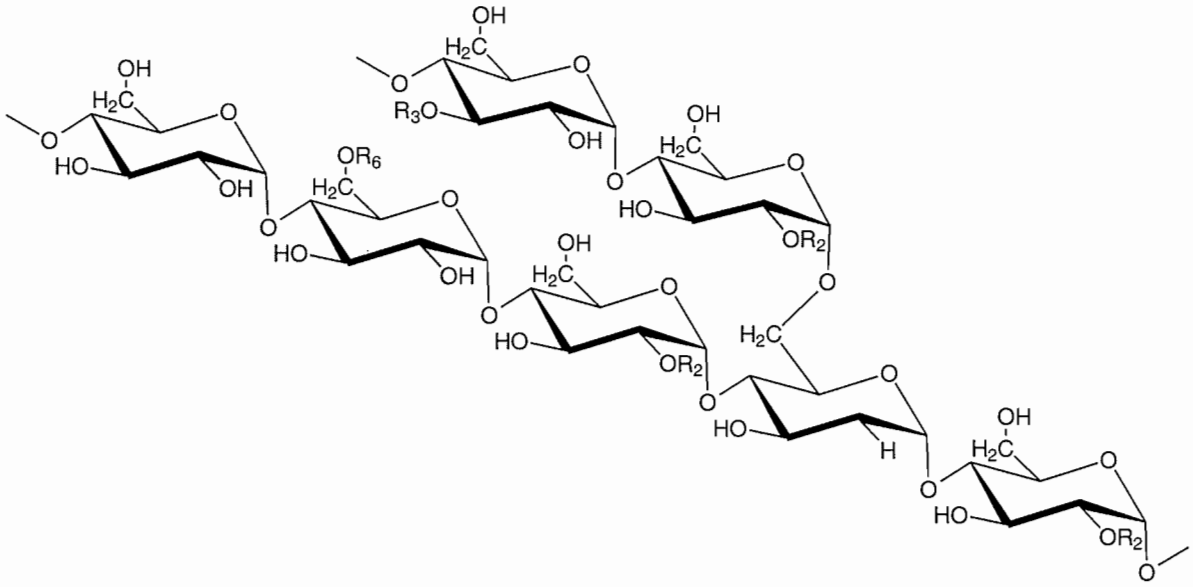


Fig. 1: Hydroxyethyl Starch

$R_2 = R_3 = R_6 = CH_2–CH_2–OH$

Experimental

For the identification of HES in human urine by GC/MS the substance is derivatised to partially methylated alditol acetates (PMAAs):

HES

Permethylation

⇓

HES permethylated

Hydrolysis

⇓

partially methylated monosaccharides

Reduction

⇓

partially methylated alditoles

Peracetylation

⇓

PMAAs

Sample Preparation for GC/MS-measurement:

10 µl of urine are placed in a tube and dried in a desiccator over phosphorus pentoxide under reduced pressure.
The residue is solved in 200 µl of dimethyl sulfoxide (DMSO) and sonicated briefly (3-5 min).

Permethylation: 200 µl of a suspension of NaOH in DMSO and 50 µl of methyliodide are added and the sample is vortexed vigorously and sonicated for 10 min.
2 ml of distilled water and 2 ml of chloroform are added and the mixture is vortexed.
After centrifugation the aqueous layer is removed and the sample is washed twice by the addition of 2 ml of distilled water each followed by vortexing, centrifugation and removal of the upper layer[10].

Hydrolysis: The organic layer is evaporated to dryness and the sample is heated with 2 ml of 3 M HCl at 100°C for 1 h.

Reduction: The sample is evaporated to dryness and 200 µl of a solution of $NaBH_4$ (5mg/ml) in a mixture of 30% methanol and 70% NaOH (0.03 M) are added. After 16 h at ambient temperature (or 4 h at 50°C) 20 µl of glacial acetic acid are added and the sample is dried by evaporation in vacuo at 50°C.
The residue is solved in 1 ml of methanolic HCl (0.1%) and evaporated to dryness.

Peracetylation : To achieve peracetylation the residue is solved with 350 µl of a mixture of acetic anhydride/ CH_3CN/ pyridine 3:3:1 (v:v:v) and heated for 2 h at 80°C.
2 ml of distilled water and 2 ml of chloroform are added, the sample is vortexed and the aqueous layer is removed.
The organic layer is dried by evaporation in vacuo and the residue is solved in 100 µl of isopropanol.

All spectra were registered on a Finnigan GCQ ion trap with the following parameters:

- Injector:	ATAS Optic 2, 300°C
- Column:	HP-5MS, filmthickness 0.25 µm, ID 0.25mm, length 18m
- Carrier Gas:	helium 1.5 ml/min, split 1:10
- Oven temperature:	1 min 80°C
	10°C/min to 260°C
	40°C/min to 320°C
- Ion source temperature:	200°C

Results

The technique of derivatising polysaccharides to PMAAs is common in sugar analysis. It enables to investigate the structure by GC/MS without losing information about linkage positions. All monomers can be detected separately and give further details about the polymer composition.

Every free hydroxy group of HES is alkylated by permethylation, even those of the hydroxyethyl groups:

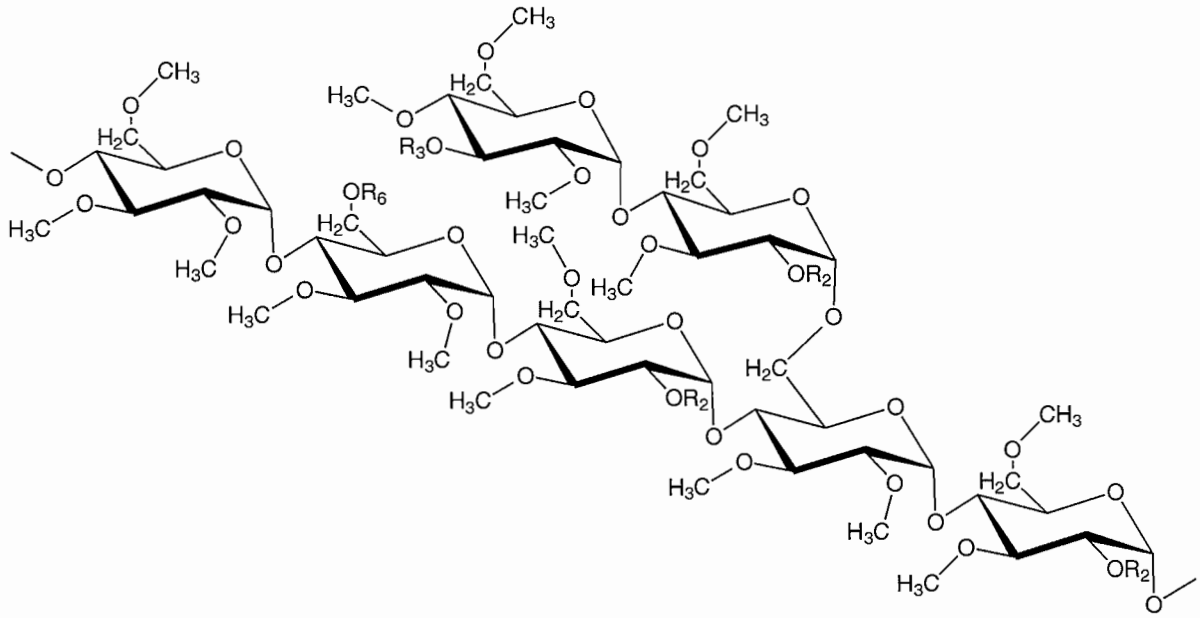


Fig. 2: permethylated Hydroxyethyl Starch

The hydrolysis of the permethylated polysaccharide cleaves the bonds between the ring structures. The resulting monosaccharides are reduced to alditoles to circumvent the fact that cyclic sugars can form two isomers because of the anomere centre C1. The free hydroxy groups of the partially methylated alditoles are acetylated and PMAAs are formed.

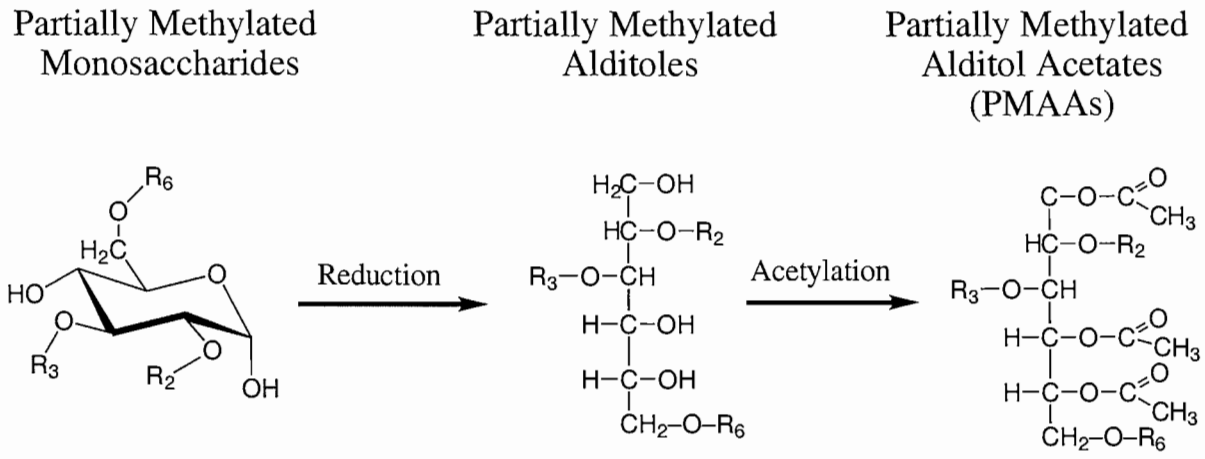


Fig. 3: Reduction and acetylation of the monosaccharides resulting from the hydrolysis of permethylated HES (R_2, R_3 and R_6 are methyl or methoxyethyl groups)

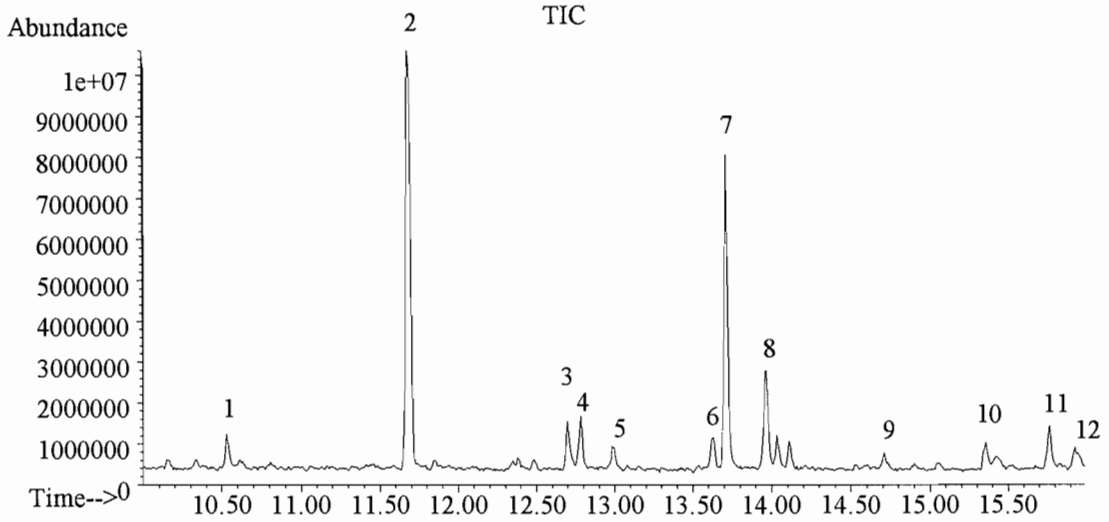
The general structure of the PMAAs shown in figure 3 allows at least four signals in a chromatogram of an HES urine sample prepared as described:

- $R_2 = R_3 = R_6 = CH_3$
- $R_2 = CH_2CH_2OCH_3, R_3 = R_6 = CH_3$
- $R_3 = CH_2CH_2OCH_3, R_2 = R_6 = CH_3$
- $R_6 = CH_2CH_2OCH_3, R_2 = R_3 = CH_3$

A typical chromatogram received from an urine sample of a patient treated with HES is shown in figure 4 (below) with an extracted ion profile of m/z 161, a selective ion appearing with most of the PMAAs of HES:

Signal 1: PMAA of free urinary glucose (1,5-Diacetyl-2,3,4,6-tetramethylglucitol)
Signal 2: PMAA of 1,4-linked glucose (1,4,5-Triacetyl-2,3,6-trimethylglucitol)
Signal 3: PMAA of terminal 2-substituted glucose (1,5-Diacetyl-2-methoxyethyl-3,4,6-trimethylglucitol)
Signal 4: PMAA of 1,4-linked glucose, uncompletely methylated
Signal 5: PMAA of terminal 3- or 6-substituted glucose
Signal 6: PMAA of HES 3 (1,4,5-Triacetyl-3-methoxyethyl-2,6-dimethylglucitol)
Signal 7: PMAA of HES 2 (1,4,5-Triacetyl-2-methoxyethyl-3,6-dimethylglucitol))
Signal 8: PMAA of HES 6 (1,4,5-Triacetyl-6-methoxyethyl-2,3-dimethylglucitol)
Signal 9: PMAA of 6-linked or uncompletely methylated HES 2
Signal 10: PMAA of HES 2.3 (1,4,5-Triacetyl-2,3-bismethoxyethyl-6-methylglucitol)
Signal 11: PMAA of HES 2.6 (1,4,5-Triacetyl-2,6-bismethoxyethyl-3-methylglucitol)
Signal 12: PMAA of 1,4-linked glucose 2-substituted by $-CH_2CH_2-O-CH_2CH_2-O-CH_3$

A)



B)

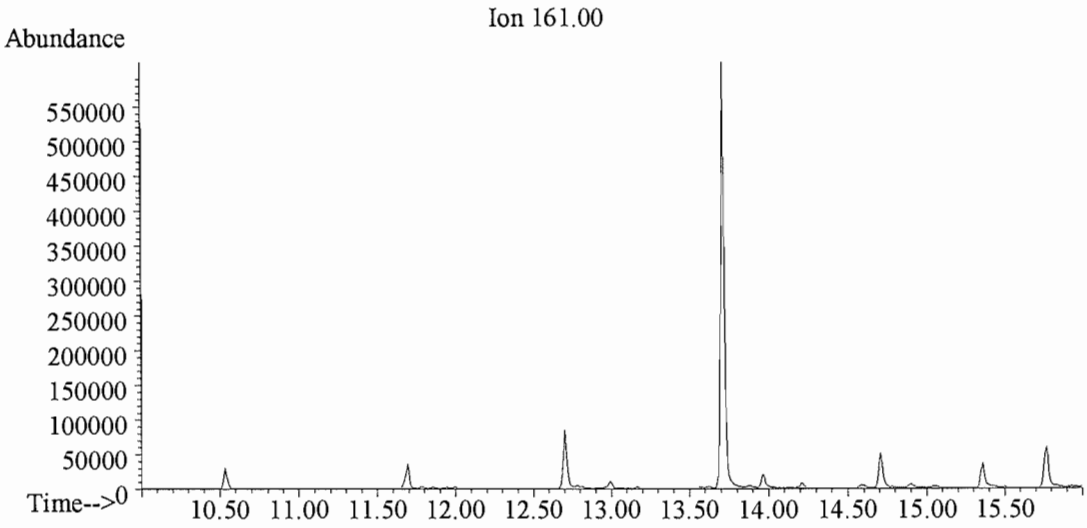


Fig. 4: GC/MS chromatogram of an HES urine sample. A) TIC, B) ion trace 161

A blank urine prepared in the same way is shown in figure 5. No signal is detected in the ion trace 161 at the retention time 13.7 min confirming the absence of HES in this urine.

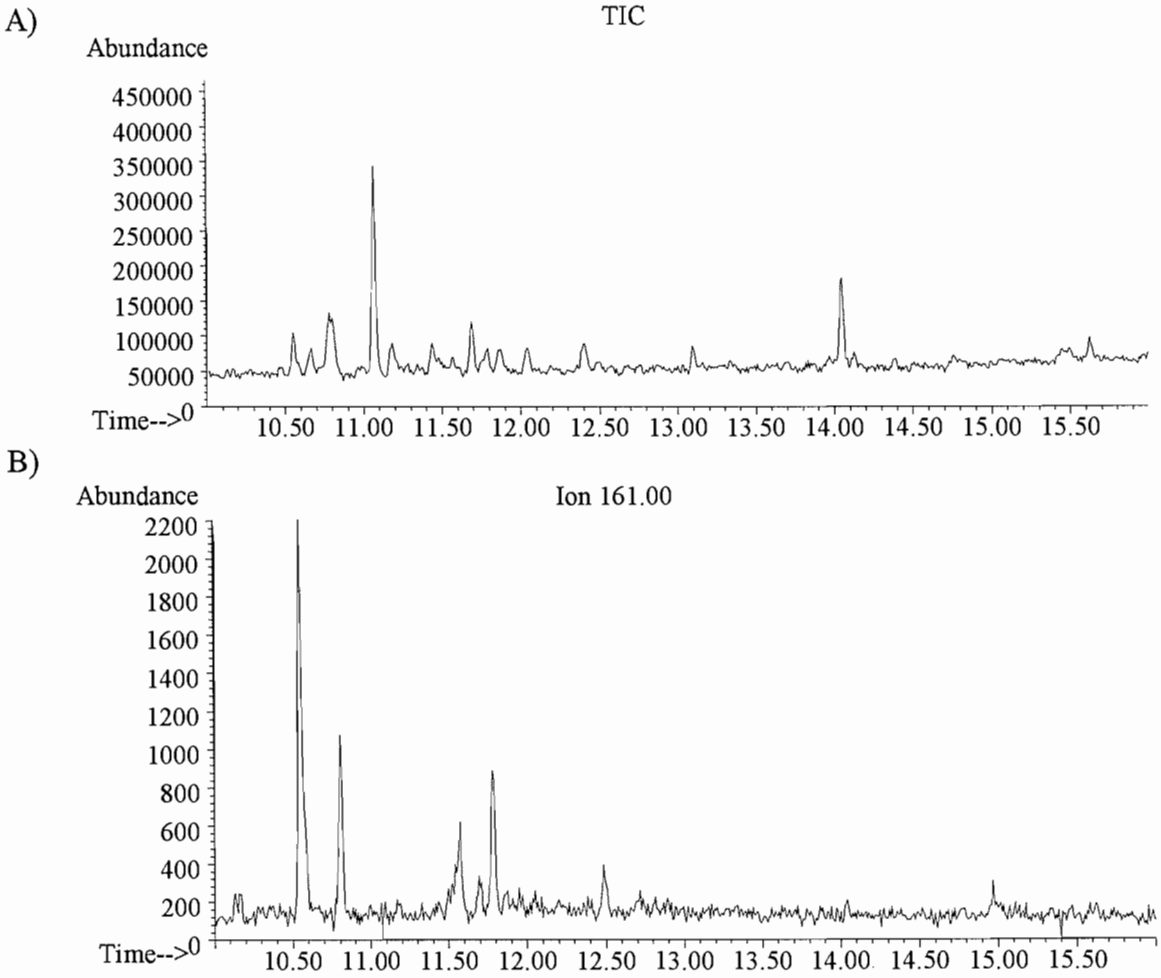


Fig. 5: GC/MS chromatogram of a blank urine. A) TIC, B) ion trace 161

Typical spectra of PMAAs resulting from HES are shown in the following:

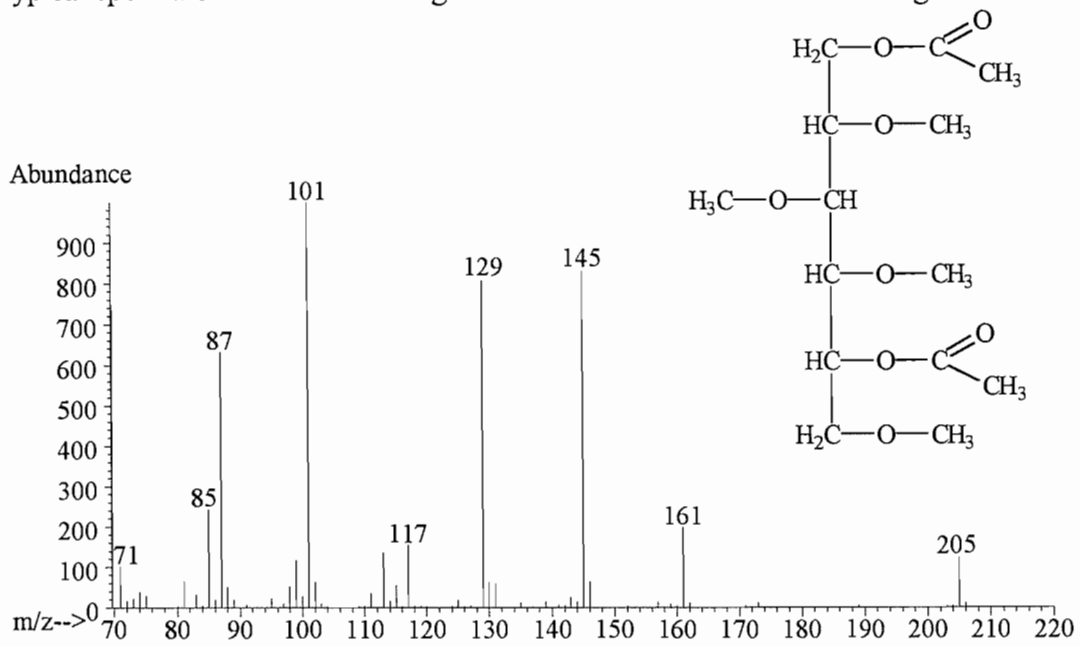


Fig. 6: EI-mass spectrum of the PMAA 1,5-diacetyl-2,3,4,6-tetramethylglucitol originating from free urinary glucose, mol wt = 322 (signal 1 in figure 4)

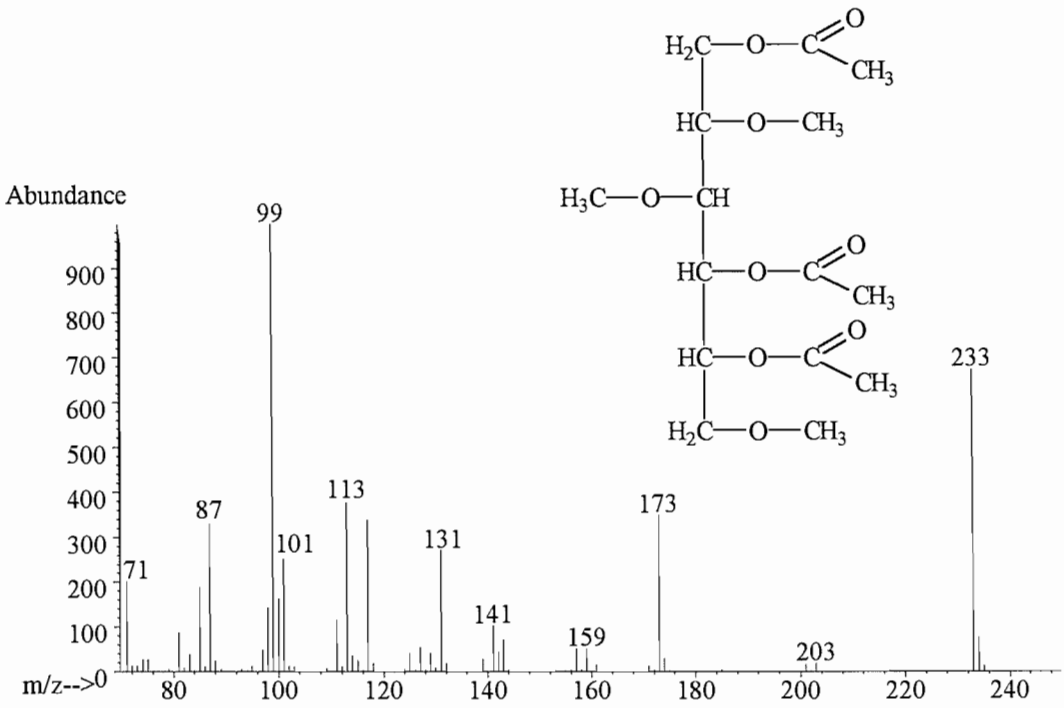


Fig. 7: EI-mass spectrum of the PMAA 1,4,5-triacetyl-2,3,6-trimethylglucitol originating from 1,4-linked glucose, mol wt = 350 (signal 2 in figure 4)

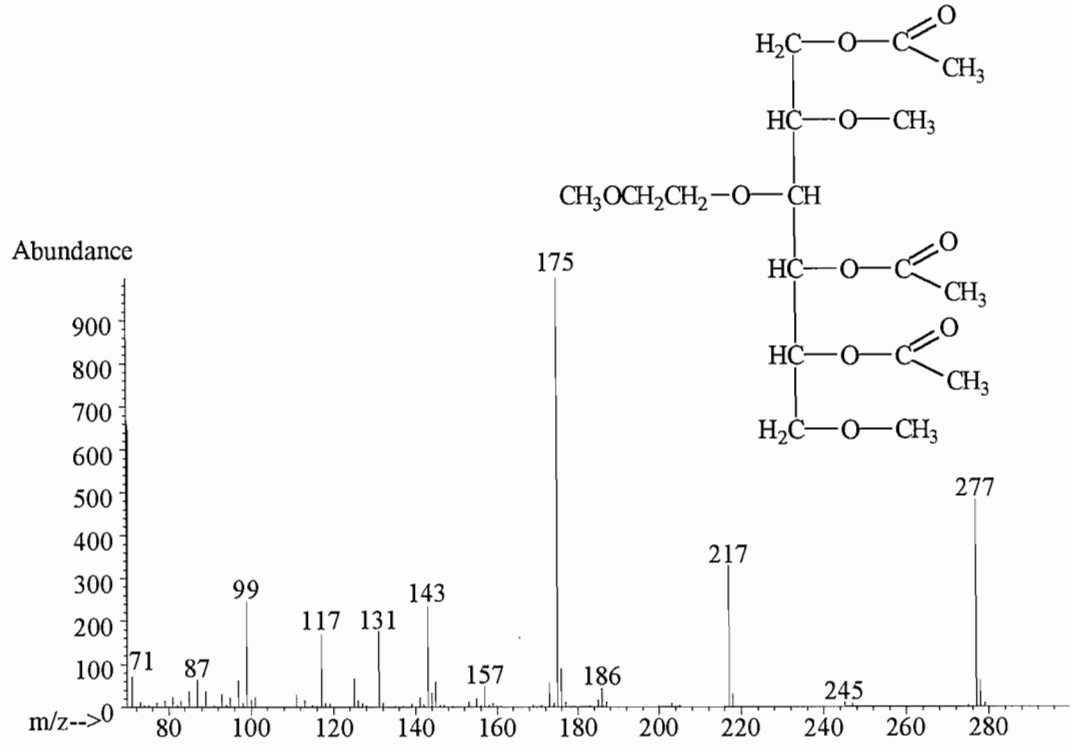


Fig. 8: EI-mass spectrum of the PMAA 1,4,5-triacetyl-3-methoxyethyl-2,6-dimethylglucitol (HES 3), mol wt = 394 (signal 6 in figure 4)

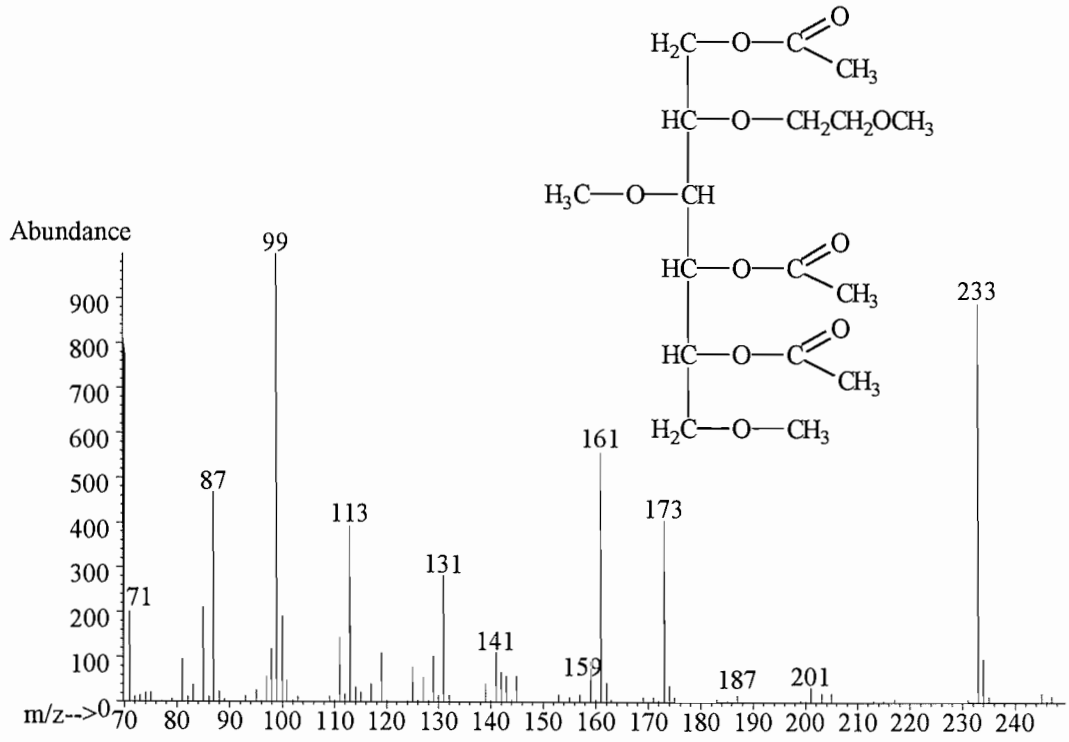


Fig. 9: EI-mass spectrum of the PMAA 1,4,5-triacetyl-2-methoxyethyl-3,6-dimethylglucitol (HES 2), mol wt = 394 (signal 7 in figure 4)

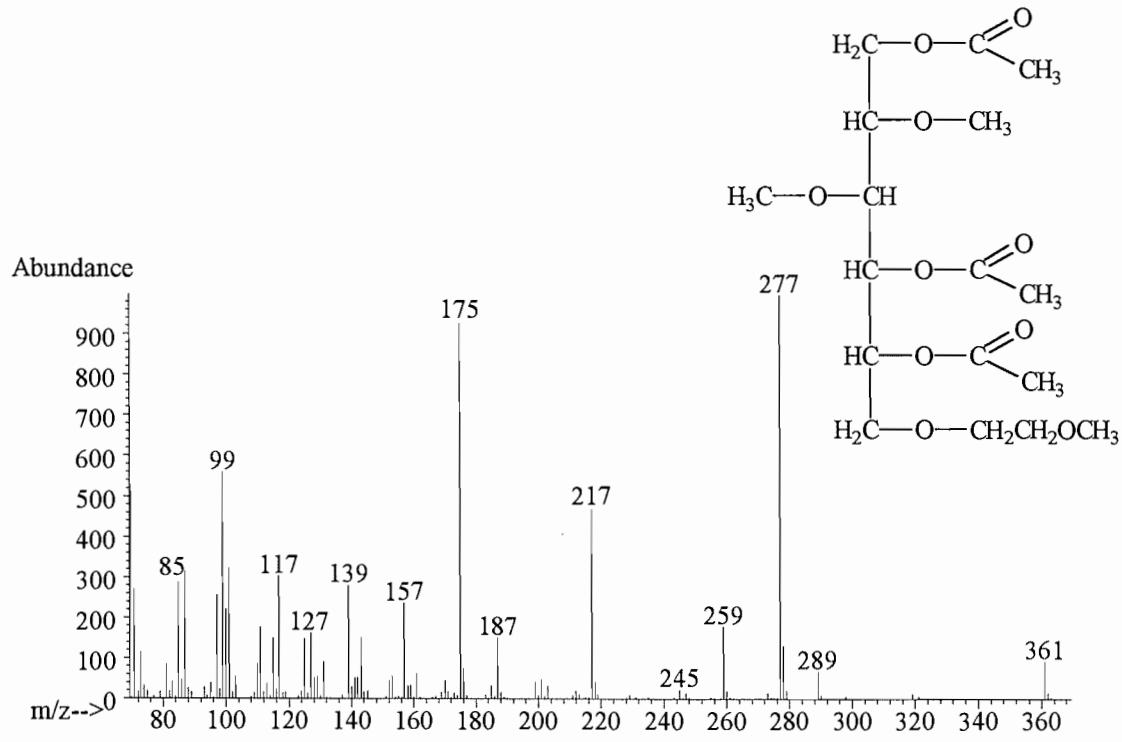


Fig. 10: EI-mass spectrum of the PMAA 1,4,5-triacety-6-methoxyethyl-2,3-dimethylglucitol (HES 6), mol wt = 394 (signal 8 in figure 4)

The presented data show one way to detect and identify HES in human urine. A useful pretest for the detection of HES in human urine is to cleave enzymatically the derivatised starch in an urine aliquot. This is performed by adding 125 U of α-amylase in a phosphate buffer (pH 6.9), heating the sample for 2 h at 37°C, adding 20 U of maltase, heating again for 2 h at 37°C and checking the glucose level by using a test strip. If there is a high amount of glucose the sample should be prepared for GC/MS. If the level of glucose is in normal ranges it is not necessary to check the urine for HES.
The PMAAs resulting from HES show spectra with comparable fragmentations. The proposed generation of some fragment ions is submitted for publication.

Acknowledgement

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References:

1 IOC doping list 1999 (January, 31th 1999)

2 FachInfo: HAES-steril® 3%/6%/10%. BPI Service GmBH, Fachinformationsverzeichnis 3 1998 (CD)

3 Mishler, J.M., Parry, E.S.: Transfusion of Hydroxyethylated Amylopectin-Protected Frozen Blood in Man. In: Vox. Sang. 36 1979 337-341

4 Burke, L.: Food and fluid intake during competition. In: Burke, L., Deakin, V. (eds.): Clinical sports nutrition. McGraw-Hill Book Company Sydney, 1994 333-364

5 Murray, B.: Fluid replacement. In: Sports Science Exchange 63 (9) 1996

6 Nitsch, E.: Volumenersatz mit künstlichen Kolloiden. In: Anästhesiol. Intensivmed. Notfallmed. Schmerzther. 33 1998 255-260

7 Yacobi, A., Stoll, R.G., Sum, C.Y., Lai, C., Gupta, S., Hulse, J.D.; Pharmacokinetics of Hydroxyethyl Starch in Normal Subjects. In: Journal of Clinical Pharmacology 22 1982 206-212

8 Kalhorn, T., Yacobi, A., Sum, C.Y.: Biliary Excretion of Hydroxyethyl Starch in Man. In: Biomedical Mass Spectrometry 11 (4) 1984 164-166

9 Brecher, M.E., Owen, H.G., Bandarenko, N.: Alternatives to Albumin: Starch Replacement for Plasma Exchange. In: Journal of Clinical Apheresis 12 1997 146-153

10 Anumula, K.R., Taylor, P.B.: A Comprehensive Procedure for Preparation of Partially Methylated Alditol Acetates from Glycoprotein Carbohydrates. In: Analytical Biochemistry 203 1992 101-108