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Determination of HES, dextran and mannitol in human urine

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Introduction

In January 2000, the International Olympic Committee added plasma volume expanders, which includes HES and dextran, to the list of prohibited substances and methods [1]. The present methods established for the detection and identification of polysaccharide-containing plasma volume expanders are based on GC-MS [2-3]. In the present study, the possibilities to analyse these polar compounds by means of LC-MS-MS are investigated and the information obtained is compared to that of the GC-MS procedure. Furthermore, the detection and identification of the osmotic diuretic mannitol should be included in both screening methods, GC-MS and LC-MS-MS, as no other screening procedure effectively covers this medical, mainly owing to its high polarity. Here, the separation of mannitol from 5 other naturally occuring stereoisomers (allitol, altritol, sorbitol, dulcitol and iditol) is necessary.

Experimental

Chemicals

The reference of dextran was Rheomacrodex® 40 (10% in 0.9% NaCl) from Pharmalink (Sweden). HETASTARCH was purchased from SIGMA. Methyliodide, dimethyl sulfoxide (dried), chloroform (for organic trace analysis), acetic anhydride, sodium hydroxide and glacial acetic acid were obtained from MERCK.

Excretion study urine samples of HES, dextran and mannitol were kindly provided by Dr. Neugebauer from the university hospital, Cologne, and Dr. Jansen from the Dominikus hospital, Düsseldorf.

Instruments

LC-MS-MS analyses were performed on an Agilent 1100 liquid chromatograph coupled to a PE Sciex API 2000 triple quadrupole mass spectrometer, equipped with an electrospray interface. All measurements were done in the positive ionisation mode. Columns used were: Merck Purospher Star 18e, 4.6 x 55 mm; Agilent XDB-C8, 4.6 x 150 mm; Macherey-Nagel Nucleosil 120-3 C18 and Macherey-Nagel Nucleosil 250-4 Carbohydrate.

The GC-MS analyses were done on an Agilent 6890/5973N instrument equipped with an HP5-MS column, 0.25 mm i.d., 0.25 μm film thickness, length 17 m. Temperature program: 140°C, +10°C/min, 320°C.

Results and discussion

It is desirable for the LC-MS-MS procedure for the detection and identification of polysaccharide-based plasma volume expanders and mannitol to include the following items:

- fast sample preparation
- short analysis time
- identification of polymeric structure
- chromatographic separation of monosaccharides (free urinary glucose, 1,4-linked glucose, 2-, 3- or 6-hydroxyethylated glucose, 1,6-linked glucose, mannitol)

The determination of a polymeric structure of the administered plasma volume expander is of paramount importance, because its persistent osmotic effect is based on its high molecular weight and the resulting extended retention in blood. In order to obtain information about a polymeric origin of analyzed monosaccharides, a permethylation of the intact analytes was performed followed by an acidic hydrolysis according to the procedure described previously[2]. The resulting monosaccharides are: 2,3,4,6-tetrakis methyl glucose (free urinary glucose), 2,3,6-trimethyl glucose (1,4-linked glucose, HES), 2,3,4-trimethyl glucose (1,6-linked glucose, dextran), trimethylated hydroxyethyl glucose (2-HEG, 3-HEG and 6-HEG) as shown in Figure 1. Mannitol

was permethylated but not stable after derivatisation under the conditions of the acidic hydrolysis. Thus, it could not be detected in this LC-MS-MS analysis.

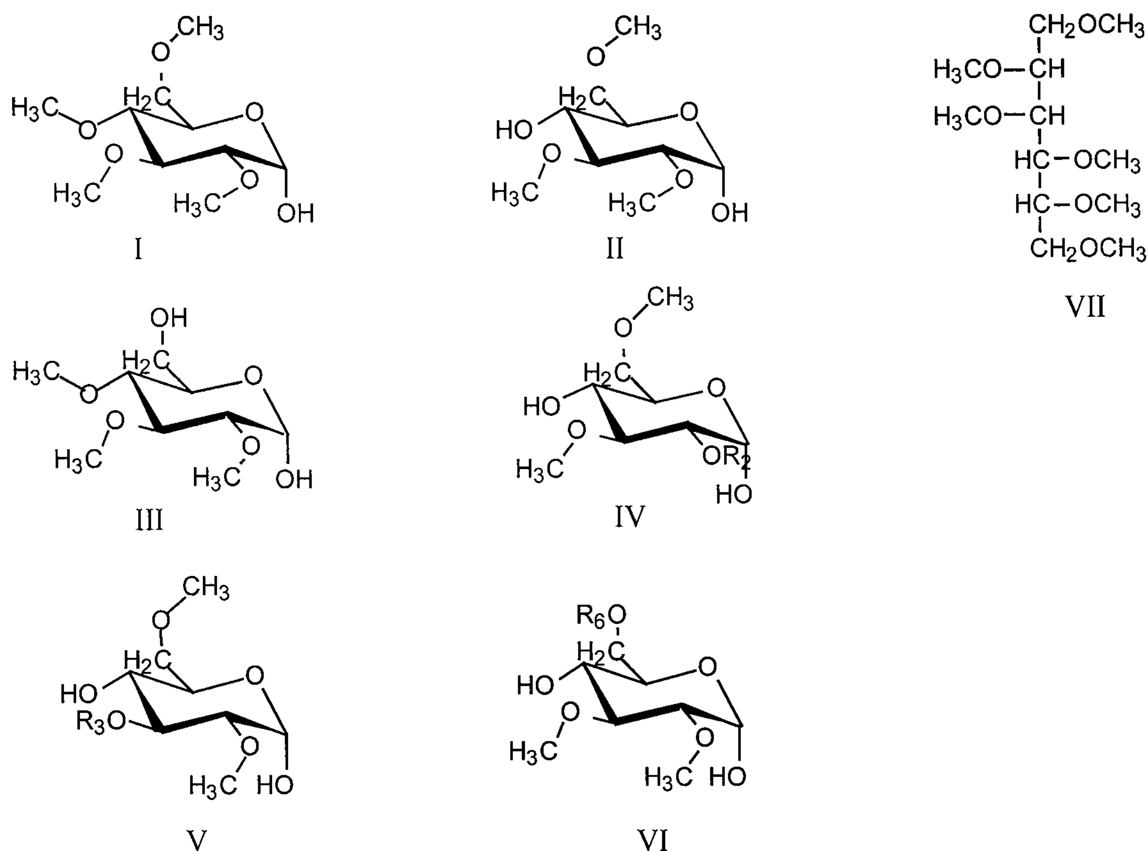

Figure 1: Analytes after permethylation and subsequent hydrolysis: I tetramethyl glucose, II 2,3,6-trimethyl glucose, III 2,3,4-trimethyl glucose, IV 2,3,6-trimethyl-2-hydroxyethyl glucose, V 2,3,6-trimethyl-3-hydroxyethyl glucose, VI 2,3,6-trimethyl-6-hydroxyethyl glucose, VII permethyl mannitol; $R_2 = R_3 = R_6 = CH_3CH_2O$

To improve the chromatographic properties of the analytes after methylation, a subsequent acetylation of the remaining free hydroxyl groups was done.

As mannitol should be included in a possible screening procedure for sugar-based compounds in human urine, the permethylation followed by an acidic hydrolysis proved to be partially inappropriate. Therefore, a method consisting of switched sample preparation steps was tested,

starting with the hydrolysis of polysaccharides followed by permethylation. Here, the resulting monosaccharides are permethylated glucose, permethylated hydroxyethyl glucose and permethylated mannitol.

In order to obtain a chromatographic separation of mannitol and its naturally occurring stereoisomers, a peracetylation was performed according to the method described previously[2]. The peracetylated derivatives could be analysed by means of GC-MS and LC-MS-MS.

Mass spectrometry

The analytes obtained after methylation and subsequent hydrolysis generate an ammonium adduct ion $(M+NH_4)^+$ after electrospray ionisation in the positive mode as shown in Figure 2 with 2,3,6-trimethyl glucose.

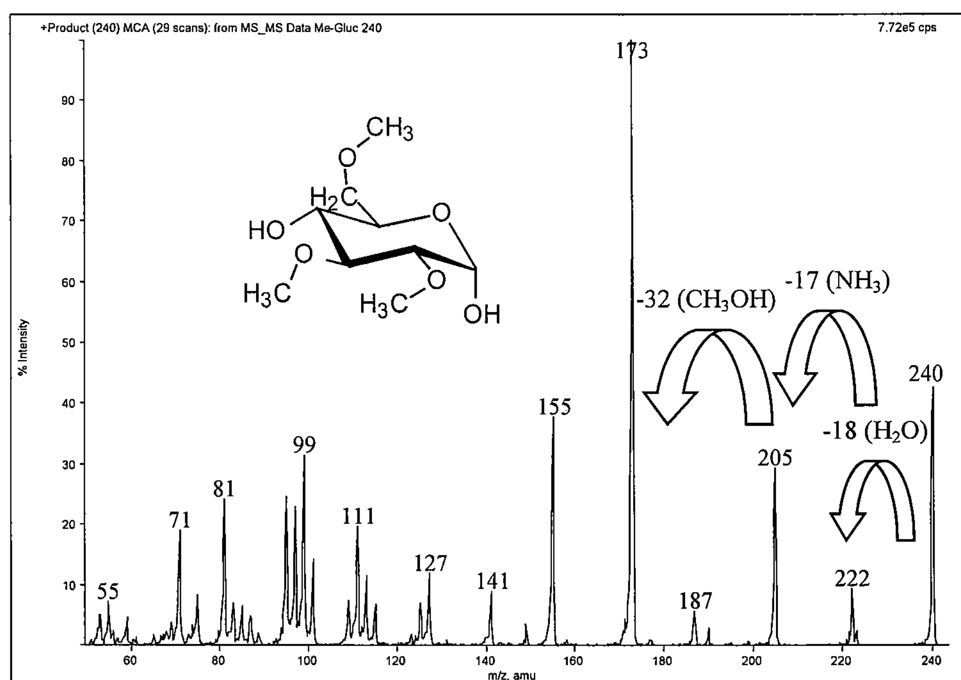

Figure 2: Product ion spectrum of *m/z* 240 of 2,3,6-trimethyl glucose (ammonium adduct)

The collision-induced dissociation gives rise to mass spectra mainly containing fragment ions generated by the neutral loss of water (-18), ammonia (-17) and methanol (-32). In case of trimethylated hydroxyethyl glucose, the removal of the hydroxyethyl side chain is observed by the elimination of 76 Da (Figure 3). The product ion spectrum of mannitol ($M+NH_4^+$ = 284)

shows a cascade of four methanol eliminations from the quasimolecular ion after removal of ammonia as shown in Figure 4.

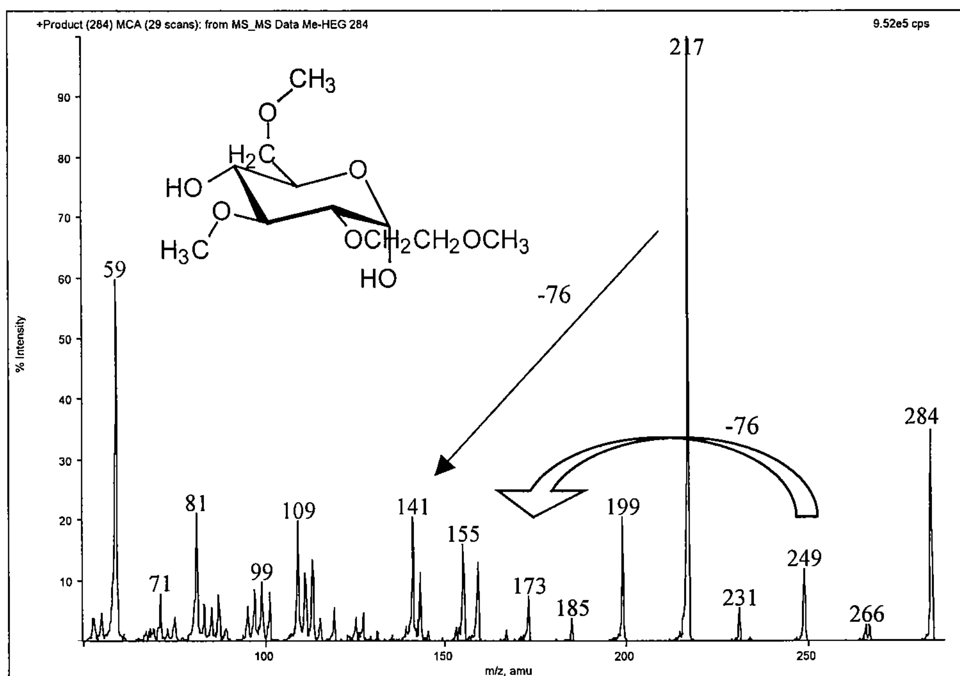

Figure 3: Product ion spectrum of *m/z* 284 of 2,3,6-trimethylated hydroxyethyl glucose (ammonium adduct)

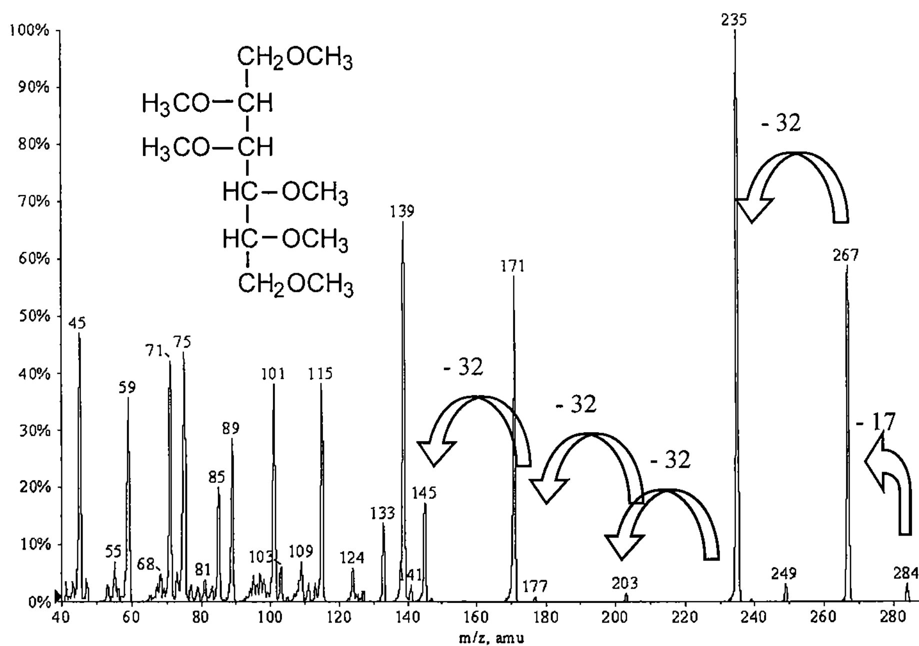

Figure 4: Product ion spectrum of *m/z* 284 of permethylated mannitol (ammonium adduct)

The additional acetylation of remaining hydroxy groups of formerly linked monosaccharides influences the fragmentation pathway by a predominant elimination of acetic acid from the quasimolecular ion, and after the subsequent loss of ammonia (Figure 5). All other losses of methanol or methylated hydroxyethyl groups are comparable to those observed without acetylation, but a significant difference was observed between the derivatives of 1,4-linked and 1-6-linked glucose. The fragment ion intensities differ in several cases intensively and furthermore, the ions 232 and 183 are present only in the product ion spectrum of the subunit of dextran, 1,6-linked glucose (Figure 6).

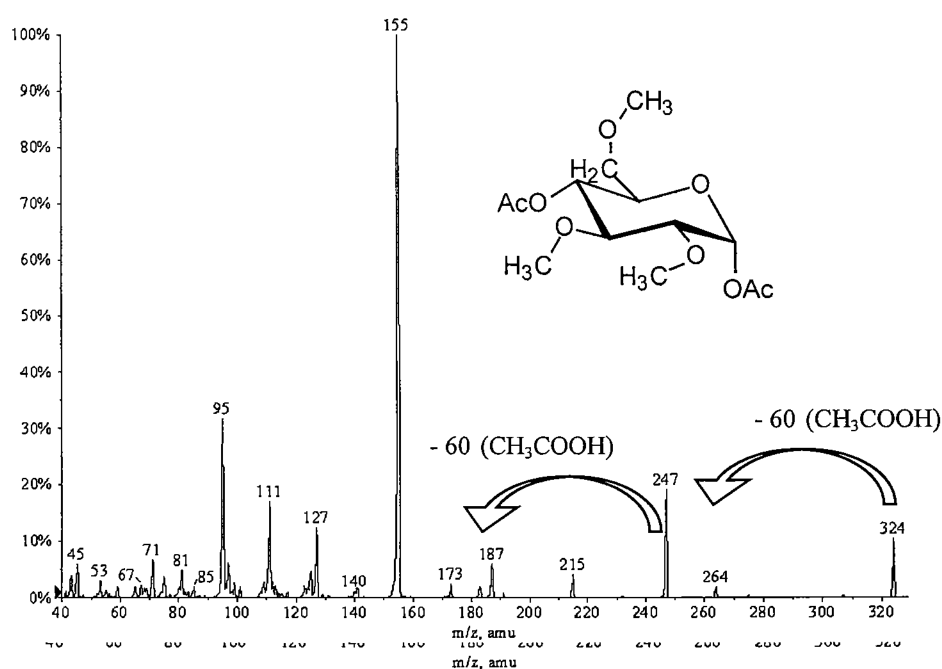

Figure 5: Product ion spectrum of *m/z* 324 of 2,3,6-trimethyl-1,4-diacetyl glucose resulting from HES (ammonium adduct)

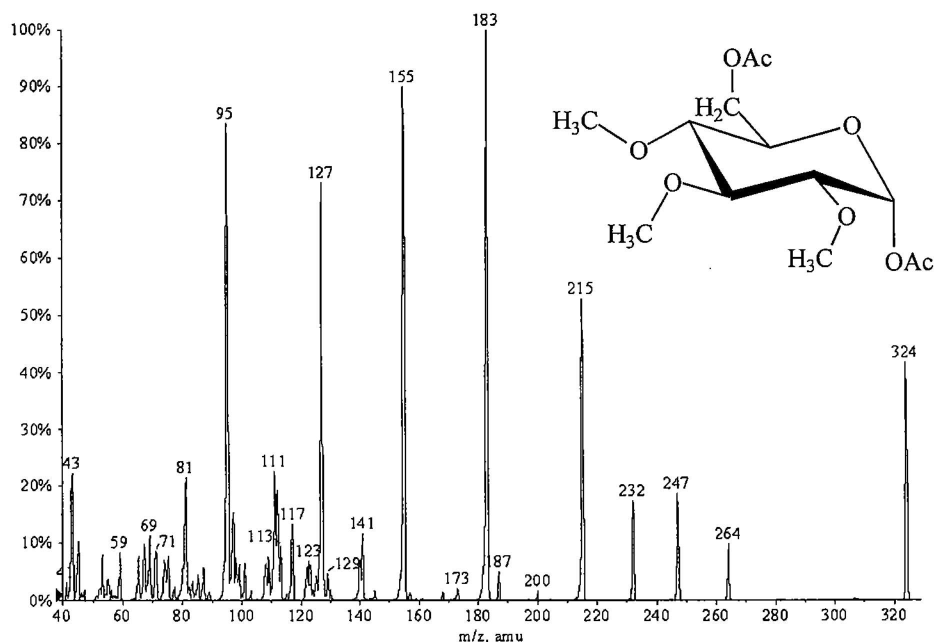

Figure 6: Product ion spectrum of *m/z* 324 of 2,3,4-trimethyl-1,6-diacetyl glucose resulting from dextran (ammonium adduct). The ions at *m/z* 232 and 183 represent a significant difference in the spectrum compared to that obtained from 2,3,6-trimethyl-1,4-diacetyl glucose (Fig. 5).

The permethylation of the monosaccharides obtained after acidic hydrolysis led to product ion spectra that mainly show neutral losses of methanol units (-32). In addition, hydroxyethylated glucose shows the expected elimination of 76 Da.

LC-MS-MS analysis

1) Permethylation and acidic hydrolysis

In Figure 7, the chromatogram of an HES excretion study urine sample is shown after treatment by methylation and acidic hydrolysis. The chromatographic properties of the partially methylated monosaccharides are only moderately good, and separation of the α- and β-isomers is not accomplished, neither of trimethylated analytes nor of the constitutional isomers 2-, 3- and 6-hydroxyethylated glucose. The identification of free urinary glucose (signals at 5.48 and 5.61 min), 1,4-linked glucose and hydroxyethylated glucose is possible. Owing to the degradation of mannitol during the hydrolysis after permethylation, we do not obtain a signal in fortified urine samples when preparing the sample by permethylation and subsequent acidic hydrolysis.

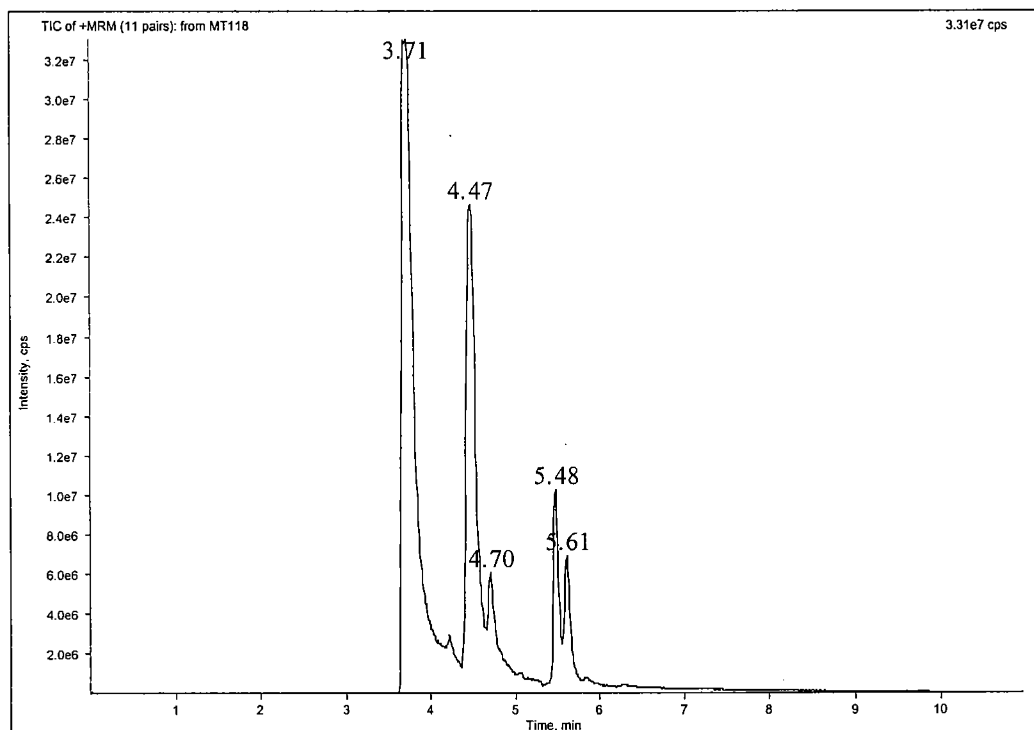

Figure 7: Chromatogram of an HES excretion study urine sample after methylation and hydrolysis. 3.71: 2,3,6-trimethyl glucose (1,4-linked glucose), 4.47 / 4.70: 2,3,6-trimethylated hydroxyethyl glucose, 5.48 / 5.61 2,3,4,6-tetramethyl glucose.

2) Permethylation, acidic hydrolysis and acetylation

In order to improve the chromatographic properties of the analytes, the remaining free hydroxy groups are acetylated and a separation of the α- and β-isomers of 1,4-linked glucose, 1,6-linked glucose and also hydroxyethylated glucose is achieved. But still, the constitutional isomers of 2-, 3- and 6-hydroxyethylated glucose are not resolved, and a signal of mannitol could not be obtained as the sample preparation still consists of methylation followed by acidic hydrolysis.

3) Acidic hydrolysis and permethylation

Starting with acidic hydrolysis, the information available from the following analysis is limited in terms of the polymeric origin of the analytes. In case of a HES excretion study urine sample, the resulting permethylated monosaccharides (glucose and hydroxyethylated glucose) are properly

separated as well as their α- and β-isomers. But still, the constitutional isomers of hydroxyethylated glucose are not resolved. Thus, the information obtained is comparable to that of the GC-MS screening procedure as we also observe an intense signal of permethylated mannitol, but the chromatographic resolution is more limited.

The identification of mannitol from a biological matrix includes the chromatographic and / or mass spectrometric separation of this analyte from other naturally occuring stereoisomers of mannitol, such as allitol, altritol, dulcitol, iditol and sorbitol (Figure 8).

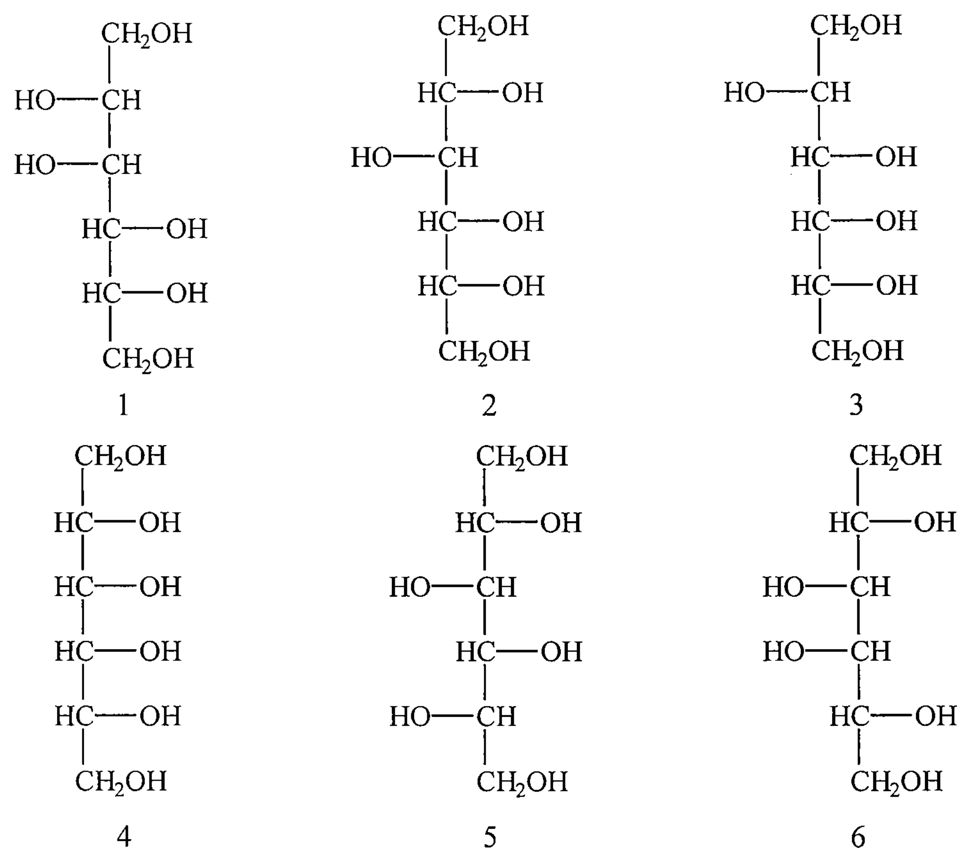

Figure 8: Stereoisomers of mannitol (1): sorbitol (2), altritol (3), allitol (4), iditol (5) and dulcitol (6)

Especially sorbitol is frequently used as a sucrose substitute and an unambiguous identification of mannitol requires its separation from its isomers. The peracetylation of the six analytes enables the measurement by means of LC-MS-MS as well as GC-MS. Liquid chromatography only provided the separation of four alditols while gas chromatography showed good resolutions of all six compounds as shown in Figure 9. Especially mannitol is baseline-separated from all other analytes, and although all isomeric alditol acetates show identical mass spectra (Fig. 10) the identification of the prohibited osmotic diuretic mannitol is possible.

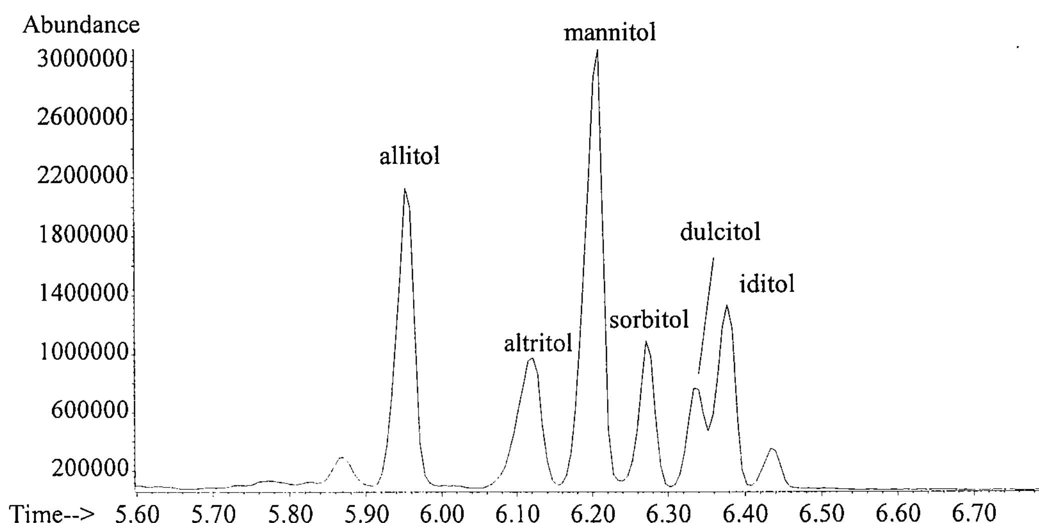

Figure 9: GC-MS chromatogram of a urine sample spiked with the reference substances of allitol, altritol, mannitol, sorbitol, dulcitol and iditol after peracetylation.

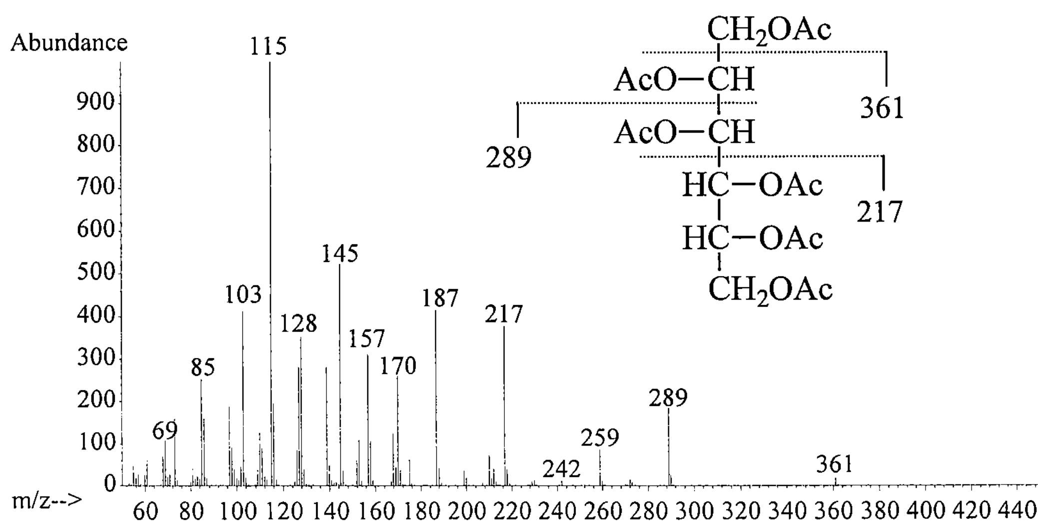

Figure 10: EI-mass spectrum of peracetylated mannitol (mol wt = 434)

Conclusion

The present studies describe a possible use of LC-MS-MS for the analysis of saccharide-based compounds relevant for doping controls. In general, the mass spectrometry after electrospray ionisation or atmospheric pressure chemical ionisation provides sufficient sensitivity and selectivity for screening and confirmation purposes of HES, dextran and mannitol. The disadvantages of the presented procedures are mainly owing to the chromatographic properties of the analytes, especially of those resulting from hydroxyethyl starch.

With methylation and subsequent hydrolysis of urine samples, partially methylated glucose derivatives are obtained that provide information about a former polymeric structure of glucose residues, urinary glucose levels and the presence of hydroxyethyl starch. No information is given about the presence of mannitol, because of its degradation after permethylation under conditions of acidic hydrolysis.

Improvements of chromatographic properties of the analytes received after methylation and subsequent hydrolysis are achieved by an additional acetylation of remaining hydroxy groups of the partially methylated glucose residues. Still, no information about the presence of mannitol can be obtained and a separation of the constitutional isomers of 2-, 3- and 6-hydroxyethylated glucose is not achieved.

A sample preparation starting with an acidic hydrolysis followed by derivatisation of the resulting monosaccharides enables the semi-quantitative detection of glucose, the identification of hydroxyethylated glucose and determination of mannitol. But here, information about a former polymeric structure is not available.

In all three cases, the sample preparation time and also analysis time is rather short, comparable to the GC-MS procedure described previously[3]. As the information about a polymeric origin of glucose residues is obtained at the expense of the detection of mannitol (sample preparation 1 and 2) and the chromatographic properties of the constitutional isomers of 2-, 3- and 6-hydroxyethylated glucose, a generic screening procedure based on permethylation followed by acidic hydrolysis proved to be impossible. The change of order of sample preparation steps (sample preparation 3) enables the semi-quantitative detection of glucose and the determination

of hydroxyethylated glucose and mannitol. Here, information about the presence of polymerised glucose is not obtained.

By means of GC-MS, unambiguous identification of mannitol is possible after peracetylation of the naturally occuring stereoisomers, chromatographic separation and mass spectrometry. With LC-MS-MS, the differentiation between four stereoisomers was accomplished.

Acknowledgements

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[2] Thevis M, Opfermann G, Schänzer W. *J. Mass Spectrom.* (2000) 35: 77-84.

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