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Detection of the plasma volume expander hydroxyethyl starch in human urine

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Abstract

The plasma volume expander hydroxyethyl starch (HES) is usually administered in cases of hypovolaemic shocks but in 1998 the press reported its misuse in endurance sports. Since January 2000, it has been put on the list of prohibited substances of the International Olympic Committee (IOC) and its misuse is to ban by doping controls. Therefore, a rapid method enabling the screening for HES in human urine was developed which can be easily adopted by IOC laboratories to analyse routine urine samples for this remedy. Excretion study urine samples obtained from patients treated with HES, blank urine specimen and reference standards, were hydrolysed with hydrochloric acid and without any further purification of the resulting monosaccharides their per-timethylsilylated derivatives were performed. By means of gas chromatography–mass spectrometry the products were separated and the α- and β-isomers of glucose, 2-, 3- and 6-hydroxyethyl glucose derivatives were identified. Typical ion traces of 2- and 3-substituted glucose (m/z 248, m/z 261 and m/z 235, m/z 248, respectively) support the fast determination of the substances whose electron impact mass spectra are presented and discussed. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Hydroxyethyl starch (HES, Fig. 1) has been investigated since 1957 as a plasma volume expanding agent with great success. In 1973 it was officially accepted in the USA, Japan and Germany for plasma replacement [1] and other medical fields discovered further applicabilities as a leukapheresis agent [2], for haemodilution in cases of disturbances in the capillary blood circulation, treatment of burns [3] and for cryoprotection of frozen stored red blood cells [4]. The preferred use of HES in comparison to other remedies in the special fields of disease treatment is due to its good tolerance because of its close similarity to human glycogen so that only weak adverse reactions and immunogenicity are observed [5]. Further, its half-life in the human body can be controlled by its degree of substitution [6] so the time of its effect can be prescribed.

Its relevance to doping was initiated in 1998 when HES was officially mentioned as being used in endurance sports. The reason for its application by athletes may originate from different advantages, e.g., the control of the haematocrit value (which is tested by different federations before an athlete gets permission to start a competition) or higher amounts

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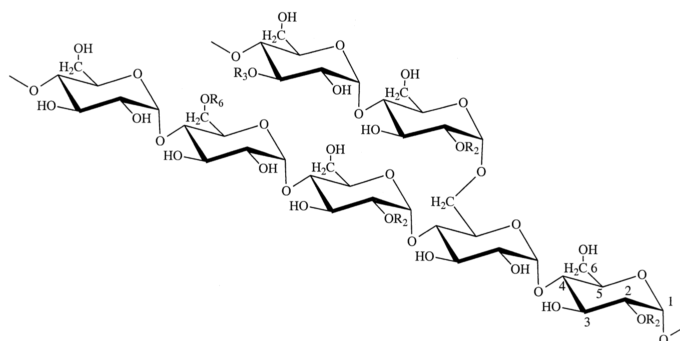

Fig. 1. Structure of hydroxyethyl starch, $R_2=CH_2CH_2$-OH at C-2 at the glucose residue, $R_3=CH_2CH_2$-OH at C-3 at the glucose residue, $R_6=CH_2CH_2$-OH at C-6 at the glucose residue.

of body fluid preventing a decrease in exercise performance because of dehydration [7,8]. Further, HES may occur in urine because of autologous blood transfusion. Since January 2000, HES is a prohibited substance according to the International Olympic Committee (IOC) doping list [9] and routine urine samples of doping controls have to be tested for possible misuse.

The heterogeneity of HES concerning the molecular mass 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 [6]. Studies showed only a recovery of less than 1% of an administered dose in faeces over a 14-day period [10], but high amounts of HES in urine [11,12] which can be detected in different ways. For confirmational analyses a suitable way of identifying HES in human urine consists of its degradation and derivatisation to partially methylated alditol acetates (PMAAs) and subsequent measurement by gas chromatography–mass spectrometry (GC–MS) [13]. With this method, detailed information about the structure and linkage positions of the polysaccharide are also obtained, but for screening purposes the sample preparation is too extensive and time-consuming. In the present report we describe a rapid and efficient way to screen for HES in urine samples of athletes.

2. Experimental

2.1. Chemicals

N-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany) and distilled before use, ammonium iodide (purum, analytical-reagent grade) was obtained from Fluka (Buchs, Switzerland), hydrochloric acid (32%) from Merck (Darmstadt Germany), ethanethiol (97%) from Aldrich (Deisendorf, Germany) and hydroxyethyl starch (HETASTARCH, 6% solution in 0.9% sodium chloride) from Sigma (Deisendorf, Germany).

2.2. GC–MS analysis

The measurements were performed on an HP 6890/HP 5973 GC–MS system from Hewlett-Packard (Waldbronn, Germany). Conditions were as follows: column: HP 5 MS capillary column, film thickness 0.25 μm, 16 m×0.25 mm I.D.; carrier gas: helium, 1.5 ml/min, split 1:10; injector temperature: 300°C; temperature program: 0 min 140°C, +20°C/min, 2 min 320°C; interface temperature: 300°C; ion source temperature: 200°C; ionisation: electron impact (EI) (70 eV); mass range: 50–550 u; sampling rate: 2.94 scans/s; scan threshold: 150.

2.3. Biological samples

The investigated urine samples were received from different sources:

(a) Excretion study urine specimen after HES application. Urine samples were obtained from the Dominikus hospital (Düsseldorf, Germany) from patients treated with HES (12–24 h after intravenous application of 500 ml of 0.9% sodium chloride solution containing 6% hydroxyethyl starch).

(b) Urine samples of high-performance athletes participating in endurance sports which were elected for doping controls.

(c) Blank urine specimens of 15 volunteers (nine males and six females aged 26–52 years).

All urine samples were stored at 4°C before preparation.

2.4. Sample preparation

A 20-μl volume of urine was placed in a grinding test tube, 500 μl of 3 *M* hydrochloric acid added, the tube sealed with a ground-glass stopper and the sample heated for 1 h at 100°C in an aluminium heating block.

After cooling to ambient temperature the mixture was dried by means of a rotary evaporator under reduced pressure (14 mmHg) at a bath temperature of 60°C and the tube was stored in a desiccator under reduced pressure (14 mmHg) over phosphorus pentoxide (1 mmHg=133.322 Pa).

2.5. Derivatisation

To the dry residue 25 μl of pyridine and 75 μl of a mixture of MSTFA–NH_4I–ethanethiol (100:0.2:0.6, v/w/v) was added, the tube sealed with a ground-glass stopper and maintained at 60°C for 20 min. The presence of NH_4I in the derivatisation mixture enabled the in-situ preparation of trimethyliodosilane (TMIS), a highly sensitive and unstable compound due to its tremendous reactivity enabling a rapid per-trimethylsilylation. After cooling to ambient temperature, 2 μl of the supernatant was injected into the GC–MS system by means of an automatic liquid sampler.

3. Results and discussion

The acidic hydrolysis of HES urine samples mainly yielded the monosaccharides glucose, 2-, 3- and 6-hydroxyethyl-glucose. These sugars were derivatised with MSTFA–NH_4I–ethanethiol to per-TMS products which were separated and determined by GC–MS measurements. Fig. 2 shows a typical chromatogram of a HES reference standard.

There are several signals which result from the derivatised monosaccharides forming two isomers for each sugar due to their anomeric centre at C-1. The signals a1 and a2 are assigned to the pentakis-TMS derivatives of α- and β-glucose, b1 and b2 to α- and β-3-hydroxyethyl-glucose (3-HEG), c1 and c2 to α- and β-2-hydroxyethyl-glucose (2-HEG), d1 and d2 to α- and β-6-hydroxyethyl-glucose (6-HEG). Typical ion traces of the derivatives of 2- and

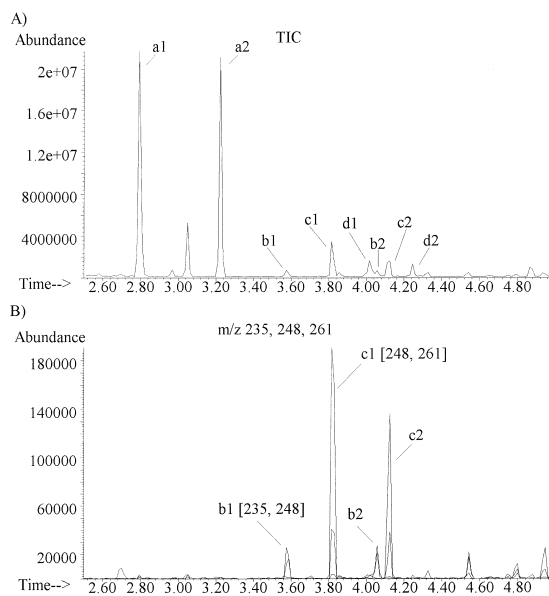

Fig. 2. GC–MS chromatogram of a HES reference standard. (A) Total ion current (TIC), a1: α-glucose pentakis-TMS, a2: β-glucose pentakis-TMS, b1: 3-hydroxyethyl-α-glucose pentakis-TMS, b2: 3-hydroxyethyl-β-glucose pentakis-TMS, c1: 2-hydroxyethyl-α-glucose pentakis-TMS, c2: 2-hydroxyethyl-β-glucose pentakis-TMS, d1: 6-hydroxyethyl-α-glucose pentakis-TMS, d2: 6-hydroxyethyl-β-glucose pentakis-TMS. (B) Selected ion traces m/z 235, 248, 261.

3-hydroxyethyl-glucose (*m/z* 235, *m/z* 248 and *m/z* 261) are also presented which are useful for a rapid determination of these substances. The full scan EI spectra of the α-monosaccharides, which are identical with those of the corresponding β-isomers, are shown in Fig. 3A–D.

The assignment of the signals in the chromatogram is based on studies investigating the EI fragmentation of the corresponding alditol acetates of HES [14] which show the same composition of HES with ca. 70% 2-substitution, 10% 3-substitution and 20% 6-substitution as is also described in the literature [15]. The summarised area % values of the corresponding α- and β-isomers of this study determined by peak integration of the total ion current (TIC) were 55.8%, 12.4% and 31.8% so the hydroxyethylated glucose derivatives could be identified, supported by typical fragment ions occurring only with 2- and 3-HEG.

Dominant fragments of the pentakis-TMS derivatives of α- and β-glucose are *m/z* 191, *m/z* 204 and *m/z* 217 (Fig. 3A) which in part are increased by values of 44 in the different hydroxyethylated compounds due to the introduction of an ethylenoxy group (as shown in Table 1). The ion *m/z* 191 is partially shifted to *m/z* 235 in 3-HEG (Fig. 3B), *m/z* 217 to *m/z* 261 in 2-HEG (Fig. 3C) and in both substances *m/z* 204 shifts to *m/z* 248. In the spectra of 6-HEG (Fig. 3D) no incremental fragments were found in comparison to the pentakis-TMS derivatives of α- and β-glucose. Only the relative intensities differ.

In comparison to other studies concerning the fragmentation of either peracetylated, partially methylated or acetylated glucose [16], the ions *m/z* 217 (*m/z* 261) and *m/z* 204 (*m/z* 248) can be described as shown in Fig. 4.

The fragment *m/z* 217 is present in all glucose derivatives (Table 1, Fig. 3A–D) and comprises the carbons 2, 3 and 4 with two TMS groups located at C-2 and C-4 (Fig. 4A). The group at C-3 is eliminated so the corresponding *m/z* 261, due to the introduction of an ethylenoxy group, can only occur in 2-HEG (Table 1, Fig. 3c). The ion *m/z* 204 (Fig. 4b) contains two carbons one of which can bear a TMS-oxy-ethylenoxy group so that the ion *m/z* 248 is observed in the spectra of 2- and 3-HEG.

The data obtained from the chromatogram com-

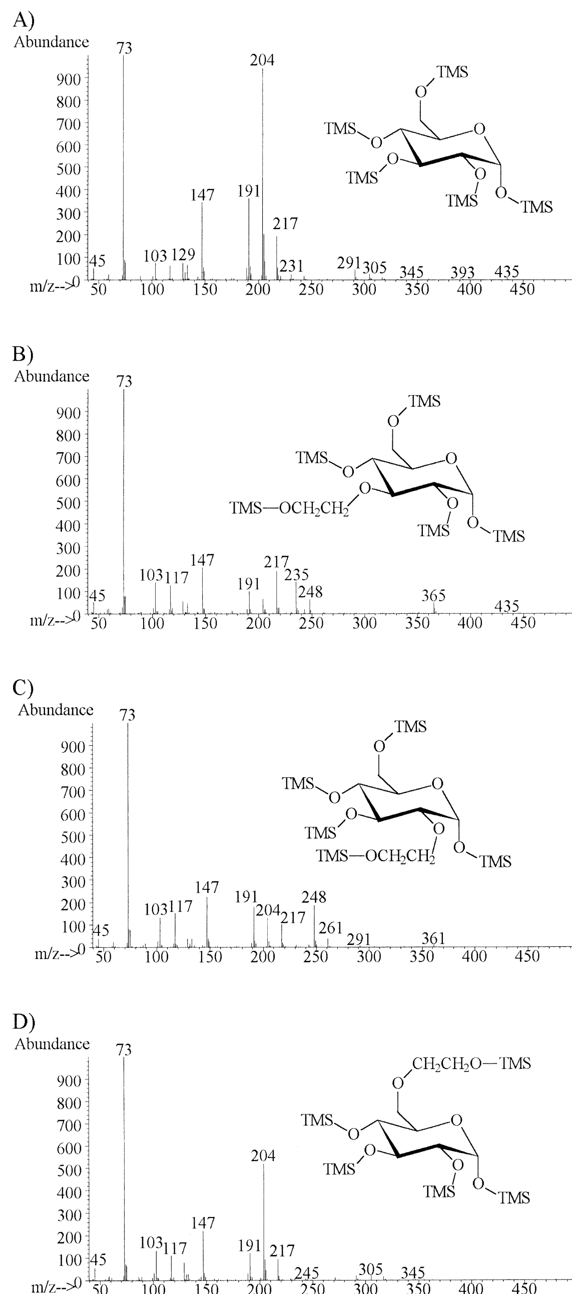

Fig. 3. (A) EI mass spectrum of α-glucose pentakis-TMS (M_r=540), signal a1 in Fig. 2. (B) EI mass spectrum of 3-hydroxyethyl-α-glucose pentakis-TMS (M_r=584), signal b1 in Fig. 2. (C) EI mass spectrum of 2-hydroxyethyl-α-glucose pentakis-TMS (M_r=584), signal c1 in Fig. 2. (D) EI mass spectrum of 6-hydroxyethyl-α-glucose pentakis-TMS (M_r=584), signal d1 in Fig. 2.

Table 1
Selected fragment ions of the pentakis-TMS derivatives of hydroxyethyl glucoses obtained after hydrolysis of HES

fragment ion m/z	Intensity in % relative to base peak (m/z 73)			
	α-glucose pentakis-TMS	α-2-hydroxyethyl-glucose pentakis-TMS	α-3-hydroxyethyl-glucose pentakis-TMS	α-6-hydroxyethyl-glucose pentakis-TMS
191	36	18	18	12
204	94	13	12	52
217	19	10	52	10
235	–	–	30	–
248	–	19	26	–
261	–	4	–	–

(+44: 191 → 235, 204 → 248, 217 → 261)

bined with ion traces and full scan spectra enable the unambiguous identification of HES in human urine as presented in the following: 15 blank urine specimens, 14 excretion study urine samples and 58 doping control urine samples of high-performance athletes were prepared and analysed as described. In 71 samples (all blank urines and 56 doping control urines), no distinct signals were observed at the expected retention times in the selected ion traces as demonstrated with an example in Fig. 5A, but the per-TMS derivative of free urinary glucose was always detected in the TIC.

All excretion study urine samples and also two of the athlete urine specimens yielded chromatograms

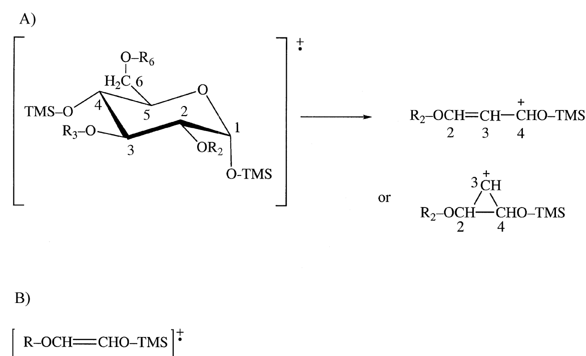

Fig. 4. (A) Proposed structure of the fragment ions m/z 217 and m/z 261. α-Glucose pentakis-TMS: $R_2=R_3=R_6$=TMS, m/z 217; 2-HEG: $R_2=CH_2CH_2O$-TMS, $R_3=R_6$=TMS, m/z 261; 3-HEG: $R_3=CH_2CH_2O$-TMS, $R_2=R_6$=TMS, m/z 217; 6-HEG: $R_6=CH_2CH_2O$-TMS, $R_2=R_3$=TMS, m/z 217. (B) Proposed structure of the fragment ions m/z 204 and m/z 248. The ion m/z 248 is formed with R=CH_2CH_2O-TMS and occurs in 2- and 3-HEG, m/z 204 results from R=TMS and is observed in all presented spectra.

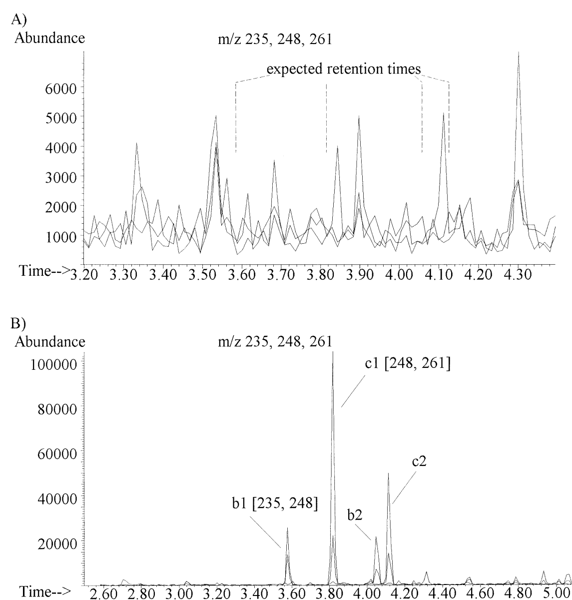

Fig. 5. (A) GC–MS chromatogram of a blank urine tested for HES. Selected ion traces m/z 235, 248, 261. (B) GC–MS chromatogram of a urine containing HES, selected ion traces m/z 235, 248, 261. b1: 3-Hydroxyethyl-α-glucose pentakis-TMS, b2: 3-hydroxyethyl-β-glucose pentakis-TMS, c1: 2-hydroxyethyl-α-glucose pentakis-TMS, c2: 2-hydroxyethyl-β-glucose pentakis-TMS.

of the ion traces m/z 235, 248 and 261 which are highly comparable to those obtained from HES standards as presented by an example in Fig. 5B.

The presence of intensive signals in the typical ion traces at the expected retention times and their mass spectra, which are identical with those received from HES reference standards, state the use of the plasma volume expander HES because hydroxyethylated glucose residues do not normally occur in human urine. A confirmation of the existence of the plasma volume expander in the urine samples can finally be done by the degradation and derivatisation to partially methylated alditol acetates as described in the literature [13,14].

The method profits from skipping the clean-up steps. The high amount of HES excreted into urine after administration reduces significantly the sample preparation time without any risk of false negative results. False positive results were not observed as proven by the confirmational analysis and in spite of different medications and the use of nutritional

supplements (as declared on sheets accompanying the doping control urine samples), no interference was observed.

All investigated urine specimen were stored at 4°C and tested for HES 1 week, 2 weeks and 4 weeks after collection. During this period the amount of free glucose in the urine increased (tested by half-quantitative glucose test strips), probably due to a degradation of HES molecules partially generating monosaccharides, while the profile of the analyte was constant in GC–MS chromatograms.

4. Nomenclature

HES	Hydroxyethyl starch
2-HEG	α- and β-2-hydroxyethyl-glucose
3-HEG	α- and β-3-hydroxyethyl-glucose
6-HEG	α- and β-6-hydroxyethyl-glucose
MSTFA	*N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide
TMIS	Trimethyliodosilane

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