

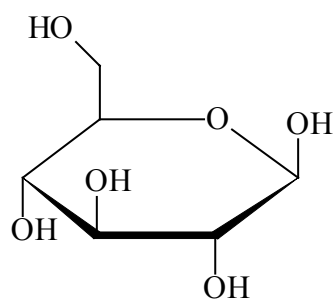
K. Deventer, P. Van Eenoo, F.T. Delbeke

## Improved clean-up for the detection of hydroxyethylstarch (HES)

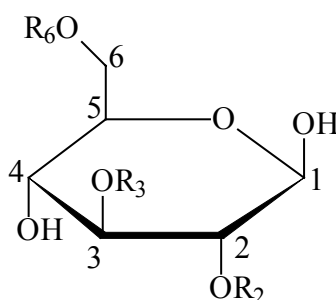
Doping Control Laboratory (DoCoLab), Ghent University-UGent, Technologiepark 30B,  
B-9052 Zwijnaarde, Belgium

### 1. Introduction

Plasma volume expanders are used for the acute treatment of shock caused by haemorrhage and burn. They are also used as priming fluids for extracorporeal units [1]. Plasma volume expanders are hypertonic solutions and pull water from the cells into the blood-vessels. In this way they increase the vascular volume and decreases cell water [2]. Hydroxyethylstarch (HES), a very popular plasma volume expander, is a corn starch derived product and consists of 1,4 polymerized glucose units substituted with hydroxyethyl-groups (Figure 1).



Glucose



2-hydroxyethylglucose :  $R_2 = \text{CH}_2\text{-CH}_2\text{-OH}$ ,  $R_3 = \text{H}$ ,  $R_6 = \text{H}$

3-hydroxyethylglucose :  $R_2 = \text{H}$ ,  $R_3 = \text{CH}_2\text{-CH}_2\text{-OH}$ ,  $R_6 = \text{H}$

6-hydroxyethylglucose :  $R_2 = \text{H}$ ,  $R_3 = \text{H}$ ,  $R_6 = \text{HCH}_2\text{-CH}_2\text{-OH}$

Figure 1: Molecular structure of glucose and hydroxyethylglucose

Commercial products are Hextend<sup>®</sup>, Pentalyte<sup>®</sup> and Voluven<sup>®</sup>.

In sports HES can be used to lower the hematocrit values in case of blood controls [3]. In sports where dehydration is common HES can be used to retain fluid to the body. As a result HES is included in the WADA list of prohibited substances [4].

Due to its high molecular weight and its polar nature the detection of HES is not straightforward.

Currently, colorimetric methods based on the detection of the polysaccharides are used for screening purposes [5-7]. However, for unambiguous confirmation mass spectrometry must be applied [8-11]. Because either enzymatic or acidic hydrolysis is used in the sample treatment for the mass spectrometrical confirmation samples with a high background level are obtained. Hence, the aim of this work was to develop an improved clean up for the samples prior to GC- or LC-MS confirmation.

## 2. Experimental

### 2.1. Chemicals and reagents

Hetastarch (6% Hydroxyethylstarch), glucose and glucose-<sup>13</sup>C<sub>6</sub> were purchased from Sigma (Bornem, Belgium). To avoid degradation of HES and glucose standards, all solutions were prepared using a mixture of water/acetonitrile (80/20).

Analytical grade methanol, hydrochloric acid (HCl) and glacial acetic acid (HAc) were purchased from Merck (Darmstadt, Germany), HPLC grade acetonitrile (ACN) was from ACROS (Geel, Belgium). Microcon-filters YM-30 (30 kDa) were obtained from Millipore (Brussels, Belgium).

### 2.2. Sample treatment

#### *Clean-up*

To remove protein- fragments 25 mg of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 200 µl of urine and the urine was vortexed during 15s. The mixture was centrifuged for 5 minutes at 10.000 rpm to precipitate the protein fragments. Then, 150 µl of the supernatant was pipetted onto a Microcon-filter YM-30 and centrifuged at 10000 rpm for 5 min. The filter was washed twice

by adding 150 µl water to the filter and centrifugation at 10000 rpm for 5 min. Finally, 150 µl water was added to the filter and the filter was centrifuged upside down at 10000 rpm (5 min) to recover the retentate (with HES) from the filter.

### *Hydrolysis*

Hydrolysis was based on a previously described method [3,10]. An internal standard solution (25 µl glucose-<sup>13</sup>C<sub>6</sub>, 1 mg/mL) and 100 µl HCl (3 M) were added to 100 µl of the retentate followed by hydrolysis of the sample at 80°C for 30 minutes. After cooling to room temperature the sample was evaporated until dry under oxygen free nitrogen (OFN) at 80 °C. To prevent corrosion by HCl during evaporation, teflon coated needles were used. The remaining residue was dissolved in 200 µl of the initial mobile phase. 50 µl was injected in the chromatographic system.

### 2.3. Validation

Ten negative urines were spiked at the threshold used for the colometric screening (i.e. 500 µg/mL) [6]. The criteria used to evaluate the presence of HES were according to the WADA recommendations.

Selectivity was tested by analysis of reference mixtures of diuretics, anabolic agents, narcotics, and stimulants. Specificity was tested by analysing the 10 negative urines which were used for spiking.

### 2.4. Instrument parameters

The HPLC system consisted of a P4000 quaternary pump equipped with a AS 3000 autosampler with a 100 µl sample-loop (all from Thermo Separation Products, Thermo, San Jose, CA, USA).

A Microsorb amine-type column 100 x 4.6 mm (3 µm) protected with a polar guard column 10 x 2 mm (both from Chrompack, Antwerp, Belgium) was used for chromatographic separation. The column was maintained at 35°C.

The mobile phase consisted of 1% HAc in water and ACN (20/80). Isocratic elution was performed at a flow rate of 0.5 mL/min (run time: 10 minutes).

Ionisation of the analytes was carried out using atmospheric pressure chemical ionisation (APCI). The capillary and vaporizer temperature were maintained at 150 °C and 350°C, respectively. The discharge current was maintained at 5µA and sheath gas and auxiliary gas were set at 80 and 10 (arbitrary units). Voltages of the capillary and lenses were optimized automatically. When MS<sup>2</sup> was applied the isolation width was set at 3.0, the activation q at 0.250 and the activation time at 30 ms.

Table 1: MS<sup>2</sup>-settings and product ions for glucose-<sup>13</sup>C<sub>6</sub> and hydroxyethylglucose in APCI negative ionisation mode.

Compound	MW	PI	CE	Product ions
Glucose- <sup>13</sup> C <sub>6</sub>	186	245	20	185
Hydroxyethylglucose	224	223	25	223, 205, 161, 143, 113

PI: Precursor Ion , CE: Collision Energy, MW: molecular weight

### 3. Results and Discussion

#### 3.1. Clean-up

HES is a macromolecule with a high molecular weight (> 100 kDa) which can not to be cleared by glomerular filtration in the kidneys. Instead, the large HES polysaccharides are cleaved by amylases in the liver. When these fragments have a molecular weight lower than 60 kDa they can be excreted through the kidneys into the urine. The presence of these high molecular weight HES molecules in the urine allowed us to use the same size exclusion filter as used for the clean-up of EPO [12]. This filter allows to remove small molecules (salts, ureic acid, ureum,...). Because the filtration of the urine with the filter also retains protein fragments with a molecular weight higher than 30 kDa a protein precipitation step with ammonium sulphate was included as well. For the precipitation the use of trifluoroic acid (TFA) and acetonitrile (ACN) were considered as well. However, the use of TFA would result in the hydrolysis of HES while with ACN precipitation of HES would occur.

### 3.2. Chromatography and mass spectrometry

Chromatography was performed on an aminopropyl type column. This kind of column is frequently used for the separation of saccharides. We also considered the use of a sugar column with embedded metals however they are expensive, operate only at high temperature and get easily deactivated.

To determine mass spectrometrical parameters for HES, the monosaccharide glucose was infused. In ESI (positive and negative mode) no ions were detected for glucose. APCI operated in positive mode didn't result in the detection of glucose neither. However, using full scan APCI in the negative mode  $[M-H]^-$  and  $[M+CH_3COO]^-$  ions could be detected. Analysis of a hydrolysed HES solution (100  $\mu\text{g/mL}$ ) resulted in the detection of 1 peak and analogous ions, i.e. taking into account the hydroxyethyl substituent. The identity of the structural isomers could not be determined due to the lack of individual reference compounds. Because full scan LC-MS spectra contain few diagnostic ions  $MS^2$  was applied on the acetate adduct of hydroxyethylglucose. The most abundant ion observed in  $MS^2$  was  $[M-H]^-$  resulting from the loss of the acetate adduct. Loss of the hydroxyethyl moiety, loss of formaldehyde and several smaller ions, resulting from the additional loss of water, were also observed.

### 3.2. Validation

The described clean-up step was a successful approach and the confirmation of HES in urine could be validated at 500  $\mu\text{g/mL}$ . As can be observed in Figure 2, a much better signal to noise ratio was obtained after applying the clean-up step.

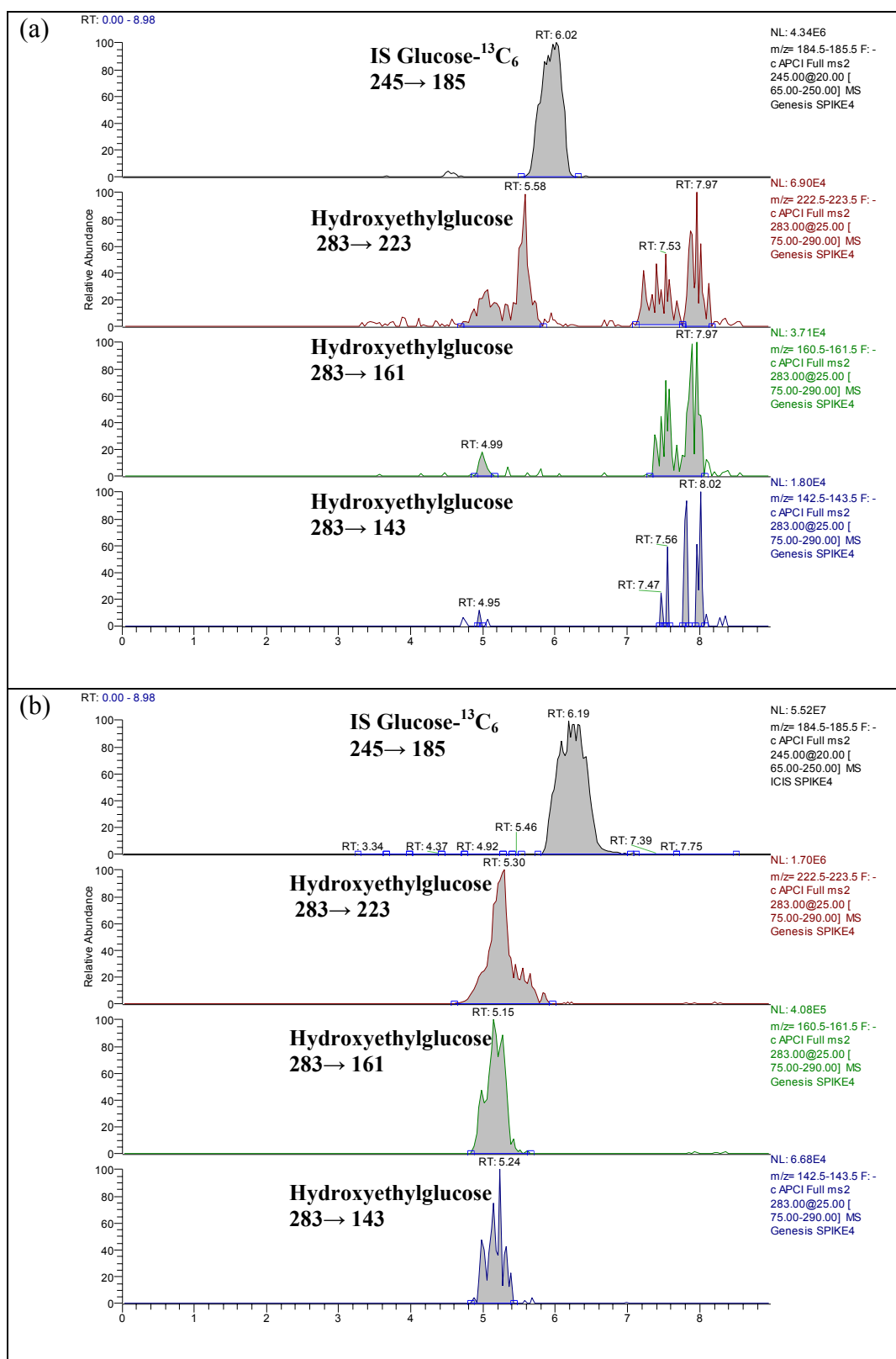


Figure 2 (a) and (b): Selected Ion Chromatograms of a urine sample spiked with HES at 500 µg/mL analysed without (a) and after applying (b) the clean-up step.

The described method is very selective as no interferences were detected when other doping agents including narcotics, corticosteroids and anabolic steroids were analysed. Specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urines were analysed. The method was also applied for the analysis of real samples (Figure 3).

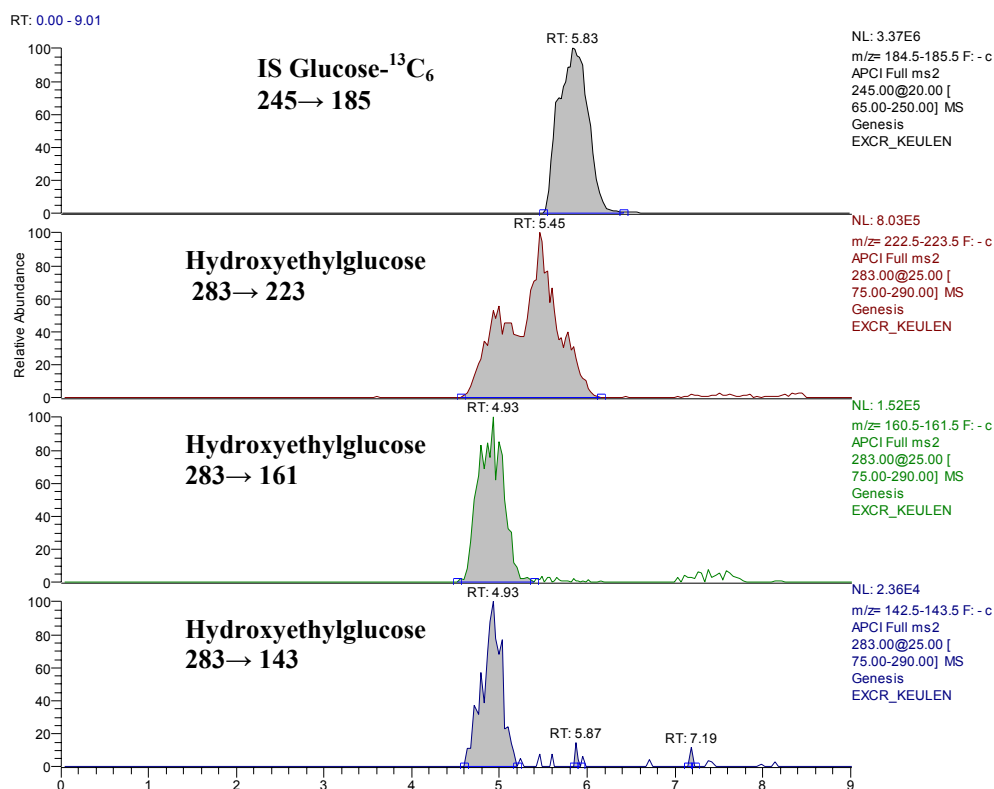


Figure 3: Selected ion chromatograms after the analysis of an excretion urine obtained from the Cologne Doping Control Laboratory.

#### 4. Conclusion

Molecular size filters used in the EPO- analysis can also be applied to clean up urine for HES analysis. This clean-up step can be applied for both the GC-MS and LC-MS confirmation methods.

## 5. Acknowledgements

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## 6. References

1. N.J. Wilkes, R.L. Woolf, M.C. Powanda, T.J. Gan, S.J. Machin, A. Webb, M. Mutch, E. Benett-Guerrero, M. Mythen, Anesh, *Anal.* 94 (2002) 538.
2. C. Christidis, F. Mal, J. Ramos, A. Senejoux, P. Callard, R. Navarro, J.C. Trinchet, D. Larrey, M. Beaugrand, C.J. Guettier, *J. Hepatol.* 35 (2001) 726.
3. M. Thevis, G. Opfermann, W.J. Schänzer, *J. Mass. Spectrom.* 35 (2000) 77.
4. WADA. 2006. The World Anti-Doping Code, The 2006 Prohibited list.
5. L. Avois, H. S. Lund, P. Hemmersbach, M. Saugy, in W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (Eds.), *Recent Advances in Doping Analysis (12)*. Sport und Buch Strauß, Köln, Germany (2004) 371.
6. R.G. Gallego, G. Such-Sanmartin, J. Segura in W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (Eds.), *Recent Advances in Doping Analysis (13)*. Sport und Buch Strauß, Köln, Germany (2005) 383.
7. D. De Boer, personal communications.
8. S. Guddat, M. Thevis, W. Schänzer, *Biomed. Chromatogr.* 19 (2005) 743.
9. M. Thevis, G. Opfermann, W.J. Schänzer, *J. Chromatogr. B.* 744(2000) 345.
10. R.G. Gallego, J. Segura, *J. Rapid Commun. Mass Spectrom.* 18 (1999) 1324.
11. K. Deventer, P. Van Eenoo, F.T. Delbeke, *J. Chromatogr. B.* 834 (2006) 217.
12. F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal. Biochem.* 311 (2002) 119.