1. Introduction

Dextran are branched polysaccharides containing a backbone of D-glucose units, mainly (~95%) in α-1,6 linkage. The remaining α-1,3 linkages account for its branching structure. Dextran solutions have been used as plasma volume expanders (PVEs), owing to surgery, burns, hemorrhage or other trauma that cause blood volume deficit [1].

Plasma volume expanders have been used by athletes mainly to control hematocrit levels and to mask blood doping with recombinant erythropoietin (rEPO) [2;3]. Therefore, they were included in the prohibited list in 2000 by the International Olympic Committee (IOC) and are maintained there now by the World Antidoping Agency (WADA) [2].

In a previous work [4] a simple colorimetric screening procedure had been proposed for hydroxyethyl starch (HES). Furthermore, a thin-layer chromatography (TLC) procedure was developed, which exhibited a characteristic profile for HES.

The objective of the present work was to demonstrate the applicability to dextran, of the previously described screening procedures. These methods reduce significantly the number of urine samples that need to be submitted to more sophisticated methodology, as it allows non-suspicious urine samples to be filtered-out.

2. Experimental

Urine samples from 4,687 athletes participating in different competitions (year 2010) were analysed by Benedict’s screening method. Urine (25 µL), diluted 10 times, was hydrolysed with 3M HCl for one hour at 100 °C and addition of Benedict’s reagent resulted in colour and precipitates, which were evaluated and interpreted. The detection limit (LOD) for dextran was obtained after spiking urine with several concentrations.
Thin Layer Chromatography (TLC) was performed and 5 µL of the hydrolysate (obtained as mentioned above) were spotted onto a silica gel 60 plate pre-coated on an aluminium sheet. Hydrolysates of urine blank, dextran solution, HES solution, isomaltose and glucose solutions were added as controls. Three consecutive runs were performed with a mixture of isopropyl alcohol, isoamylic alcohol and ammonium hydroxide (24:6:10, v/v/v). Bands were visualized using 25 mL of a 2 mg/mL solution of orcinol in 20% H₂SO₄ sprayed onto the plate and heated at 180 °C for approximately two minutes [4].

Liquid chromatography (LC) coupled with tandem mass spectrometry (LC-MS/MS) was performed with direct injection of 20 µL of Benedict’s method hydrolysate, diluted 4 times in mobile phase (acetonitrile:water, 1:1, containing 0.1% formic acid and 5.5 mM of ammonium formate) into the chromatographic systems.

The LC-MS/MS system consisted of a Varian MS-pump, Agilent autosampler and one Zorbax SB C-18 column (150 mm x 2 mm, 5 µm) from Agilent Technologies (Agilent, Palo Alto, CA, USA). The column was maintained at 40 °C during analysis. Mobile phase A (mpA) consisted of an aqueous solution of 0.1% formic acid and 5.5 mM of ammonium formate, and mobile phase B (mpB) consisted of acetonitrile containing 0.1% formic acid. A gradient elution at a flow rate of 1.0 mL/min was performed: 1 min – 45% B, 3 min – 85% B, 5 min – 85% B, 5.5 min – 45% B and 7 min – 45% B. Total run time was 7 min per sample. The LC effluent was pumped into an Applied 5500 mass spectrometer system hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with an ESI source (operated at 4500V), in the positive ion mode. Q3 was set to unit resolution in MRM mode. Nitrogen was used as nebulizer and curtain gas. The flows were set at 40 psi and 15 psi, respectively, and the capillary temperature was 750 °C.

<table>
<thead>
<tr>
<th>Target Compound</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Dwell (ms)</th>
<th>DP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>181</td>
<td>106</td>
<td>50</td>
<td>51</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>342</td>
<td>325</td>
<td>50</td>
<td>60</td>
<td>102</td>
<td>12</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>505</td>
<td>325</td>
<td>50</td>
<td>101</td>
<td>129</td>
<td>16</td>
</tr>
<tr>
<td>Isomaltotetraose</td>
<td>667</td>
<td>325</td>
<td>50</td>
<td>61</td>
<td>114</td>
<td>16</td>
</tr>
<tr>
<td>Isomaltopentaose</td>
<td>829</td>
<td>325</td>
<td>50</td>
<td>196</td>
<td>340</td>
<td>26</td>
</tr>
<tr>
<td>Isomaltohexaose</td>
<td>991</td>
<td>325</td>
<td>50</td>
<td>96</td>
<td>114</td>
<td>14</td>
</tr>
</tbody>
</table>

The MRM transitions using Q1 and Q3 masses and collision energy values were determined for individual compounds (obtained from dextran standard hydrolysates) by direct infusion into the mass spectrometer. Spectrometry conditions are summarized in table 1.
3. Results and discussion

Regarding Benedict’s screening method, the resulting urine solutions were evaluated as follows: blue limpid solutions = negative for dextran (or HES and other reducing sugars); greenish cloudy solutions, very cloudy greenish or green solutions with yellow or brick precipitate = suspect for dextran (or HES and other reducing sugars). The LOD for dextran was found to be 1.92 mg/mL. The LOD for HES was found to be 4.8 mg/ml [4].

The described Benedict’s method was applied for screening of a total of 4,687 urine samples from athletes, of which 101 (2.15%) were considered “suspect”. When these suspect samples were submitted to TLC together with controls, no typical patterns were found, resulting in no false positives to be confirmed (Fig 1).

Figure 1: All lanes, except lane 2, represent hydrolyzed samples. Lane 1, urine blank; lane 2, unhydrolyzed 0.6% dextran solution; lane 3, 0.6% dextran solution; lane 4, 0.6% HES solution; lane 5,”suspect” urine; lane 6, isomaltose standard; lane 7, glucose standard.

It must be reminded that total acid hydrolysis of dextran yields only glucose, while partial hydrolysis yields mainly glucose, and depending on the hydrolysis conditions, isomaltose and higher homologs.

The characteristic TLC profile obtained from dextran solutions submitted to acid hydrolysis during one hour at 100°C showed a strong band at the glucose level, a well defined band below glucose, identified as isomaltose, and a diffuse weak band around the application point, which represents higher isomaltose homologs. Thus, this characteristic pattern may suggest the presence of dextran in the urine sample. These results were confirmed by LC-MS/MS (Fig. 2).
4. Conclusion

The screening method based on Benedict’s reaction and the subsequent TLC method are efficient, reliable and useful procedures to limit the number of urine samples that need to be submitted to sophisticated methodology. Being simple, reproducible and low-cost, they represent economy of time, equipment, material and qualified staff. It must be stressed that these methods allow screening for dextran and HES, using only a small amount of a single urine specimen. Thus, a single thin layer chromatogram can suggest misuse of 2 different prohibited PVEs, dextran and HES, by revealing their characteristic patterns. The usefulness of the described screening methods is evident. Out of 4,687 examined urine samples, no false positives needed to be confirmed by sophisticated analyses, as Benedict’s reaction filtered-out 97.85% of the samples and TLC, the remaining 2.15%.

Acknowledgements: FUJB, FAPERJ, CNPq, CBF.

References: