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A MICROTITER ASSAY TO DETECT THE PRESENCE OF GLUCOSE-BASED PLASMA VOLUME EXPANDERS IN URINE

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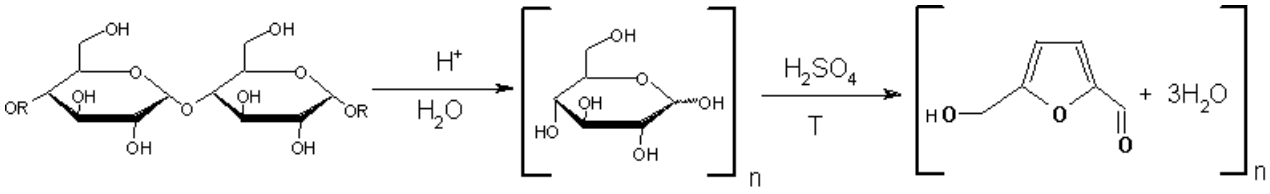
Introduction

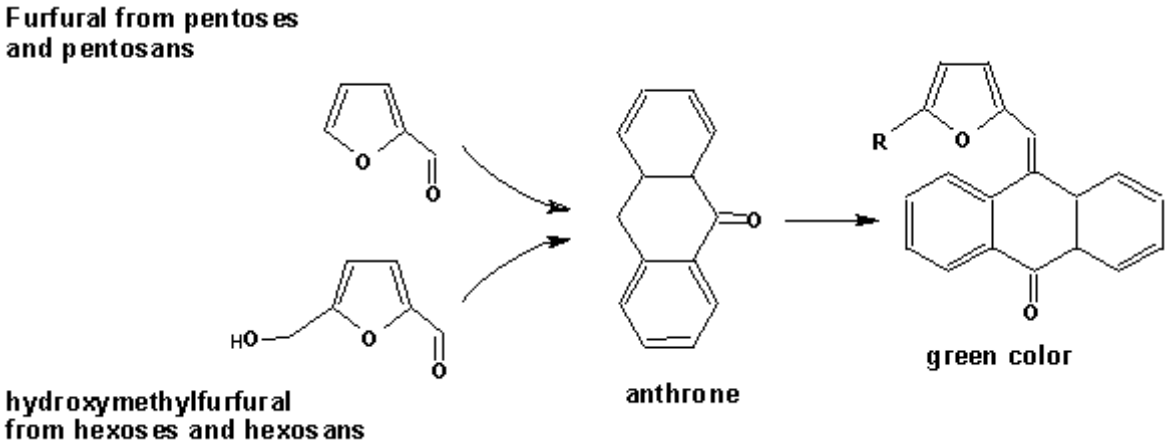
Hydroxyethyl starch (HES) is one of the most frequently used plasma volume expanders (PVE) in the medical field due to its limited side effects[1,2]. It may also be employed by athletes to prevent dehydration as well as to mask erythropoietin (EPO) abuse. Therefore HES was included in 2000 into the list of banned substances by the International Olympic Committee (IOC)[3] and in 2004 by the World Antidoping Agency (WADA)[4]. Currently, there are three well-documented methods to detect the presence of HES in urine, two based on gas chromatography and mass spectrometry[5,6], and one based in MALDI-TOF measurements[7]. All methods are fairly laborious and only a limited number of samples can be processed in batch-wise set-up. Thus, a simple and high-throughput screening protocol to filter-out non- suspicious samples would permit concentrating efforts on those really worth analysing. Attempts in this direction were presented last year by Avois *et al.*[8]. Here we present a protocol based on a microtiter colorimetric assay (MCA) for glucose-based polysaccharides[9]. It relies on the conversion of polysaccharides into monosaccharides through acid hydrolysis and the conversion of monosaccharide-derived furfurals into a UV-absorbing complex with anthrone. Measurements are performed in a microtiter plate allowing processing of up-to 96 samples in 30 minutes.

Procedure

Theoretical background – Acid-catalysed hydrolysis of polysaccharides followed by a triple dehydration of the monosaccharides to yield [5-(hydroxymethyl)]-2-furaldehydes that



subsequently react with anthrone to give a UV-absorbing complex (620 nm)



Acid hydrolysis, derivatisation and readout – In order to generate a calibration curve dextran-70 is used. Five different concentrations (0, 75, 125, 175, and 225 µg/ml) are employed. As negative control 100 µl of blank urine is diluted with 300 µl of water. As positive control 100 µl of PVE containing urine diluted with 300 µl of water is employed. Samples are diluted in a similar fashion; 100 µl of sample urine with 300 µl of water. The anthrone reagent solution is prepared freshly, just before the incubation (10 mg of anthrone in 5 ml concentrated H_2SO_4 (2 g/l), kept in the dark at 4ºC).

Procedure – **1.** Pipette 40 µl of the solutions as mentioned above to individual wells. **2.** Add 100 µl of anthrone solution to each well. **3.** Shake the microtiter plate for > 2 min. **4.** Seal the plate with acetate-tape plate sealer. **5.** Incubate at ~70 ºC (water bath) for 10 min. **6.** Cool the microtiter plate to room temperature in a cold water bath. **7.** Dry the plate (tissues). **8.** Read absorbance at 620 nm. 9. Data analysis.

Results

The MCA results typically in a differentially coloured reaction product as a function of the glucose content of the sample. Figure 1 displays a representative example. In the case that no glucose or glucose-containing polymer is present in urine the reaction product is bright yellow (un-reacted anthrone). However, the presence of glucose yields a green coloured complex absorbing at 620 nm.

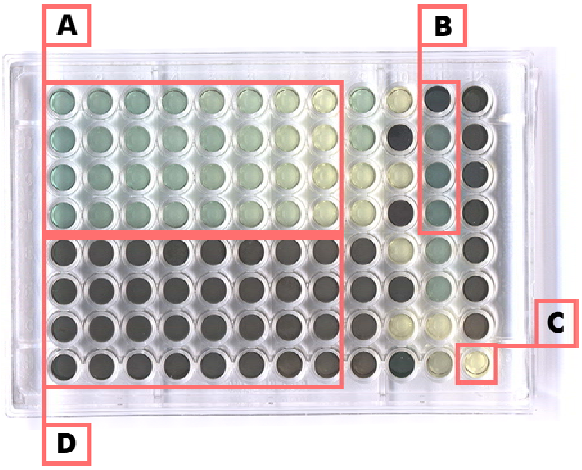


Figure 1. Microtiter coulorimetric assay (MCA) for glucose. **Block A:** Four replicates of a dextran calibration curve (9 different concentrations). **Block B:** Various urine specimens, known to contain HES (both from excretion studies and hospitalised patients). **Block C:** Blank urine specimen. **Block D:** Same as Block A with blank urine as matrix. In spite of visual differences, read-outs at 620 nm show identical results.

The absorbances can be quantitated through a calibration curve. This curve was set-up centred around a glucose concentration of 500 µg/ml, a value chosen in view of studies on the glucose concentration in urine after administration of PVE and in the absence of pathology. An example of a calibration curve (o) including five dextran concentrations and five real samples is provided in figure 2. Samples (x) devoid of glucose yield low absorbance values (< 0.1) and are readily distinguished from glucose containing samples (x). The latter are then selected for true PVE analysis based on GC-MS[5,6].

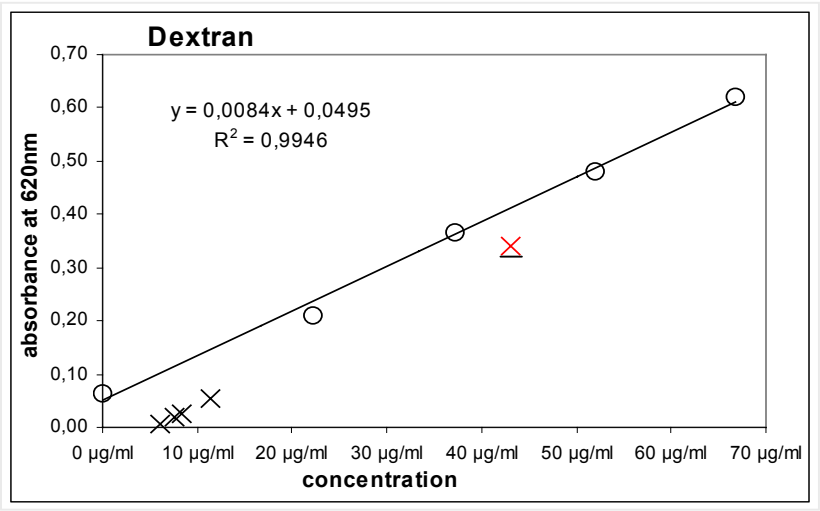


Figure 2. Calibration curve for Dextran-70 (O) and real samples (×).

Both GC-MS and MALDI-TOF[7] analysis of this batch confirmed the absence of PVE in the MCA-negative samples. The MCA-suspicious sample in this particular batch turned out to contain an elevated level of saccharose.

Table I. Quantification of the glucose levels of the samples depicted in figure 2.

SAMPLES	Absorbance	[urine] (calc)	result
urine sample 1	0,020	106,407 µg/ml	**Negative**
urine sample 2	0,341	603,854 µg/ml	**Suspicious**
urine sample 3	0,054	159,096 µg/ml	**Negative**
urine sample 4	0,006	84,711 µg/ml	**Negative**
urine sample 5	0,027	117,255 µg/ml	**Negative**

Conclusions

The microtiter assay (MCA) presented here can be employed to rapidly discriminate between glucose-containing and glucose-free specimens. Glucose levels in urine should be ~ 0 g/l in healthy individuals. Levels above this value are indicative of a renal failure or the presence of glucose containing PVE[10]. In order to select samples for further, specific analysis, a threshold level of 500 µg/ml was set. This value was selected in view of glucose levels encountered in urine after HES administration.

The method is fast, allowing simultaneous analysis of up-to 96 samples (including controls) within 30 minutes. The approach is general as PVE, other than HES, such as dextran or acetyl starch are also covered. A specific analysis of glucose-containing specimens is still required as free glucose in urine could be present for other reasons.

Literature

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