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L. AVOIS, H.S. LUND, P. HEMMERSBACH, M. SAUGY:
Rapid Screening of HES in Urine with Colorimetric Detection
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Rapid screening of HES in urine with colorimetric detection

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Introduction

Hydroxyethyl starch (HES) solutions are artificial colloids for intravascular volume replacement derived from corn starch amylopectin. HES solutions are polydisperse, comprising a distribution of molecular sizes. The main characteristics of a HES type are mean molecular weight, molar substitution (mol hydroxyethyl residues per mol glucose subunits), and C₂⁴/C₆ ratio (the substitution pattern at the glucose subunit carbon atoms). HES solutions are highly effective plasma expanders used for the treatment and prevention of hypovolemia, for acute normovolemic hemodilution, for the treatment of burns and as cryopreservative.

![Chemical structure of HES](attachment:image)

The misuse of HES in high performance endurance sports was officially mentioned in 1998 and, since January 2000, the Medical Commission of the International Olympic Committee (IOC) and more recently the World Anti-Doping Agency (WADA) prohibit the use of any plasma volume expander. Because of its various properties, HES is used by some athletes to control their haematocrit or hemoglobin value (which are tested by different federations before an athlete gets permission to start a competition) or to increase body fluid amounts to prevent a decrease in exercise performance due to dehydration. It can be noticed that the administration of plasma expanders as HES has been associated with unwanted side effects, such as platelet dysfunction, inhibition of coagulation factors, excessive intravascular volume,
pyrexia, itching, metabolic acidosis and anaphylactic reactions. Consequently, routine urine samples from doping controls are daily tested for possible HES misuse.

A GC-MS method for the screening and confirmation of HES in urine was developed four years ago, but the sample preparation is quite extensive and time consuming [1,2]. Indeed, the urinary sample undergoes an acidic hydrolysis and a derivatization step to allow identification of the various monomer units with their corresponding typical fragment ions. Therefore, the aim of this study was to develop and improve a rapid and easy screening method for the detection of HES in urine with I$_2$/I$_2$-reagent and colorimetric detection at 490 nm. Good performance results were obtained and an excretion study was evaluated with the validated colorimetric method.

**Experimental**

**Preparation of iodine solution:** 2 g of iodine and 3 g of potassium iodide were dissolved into 100 ml of distilled water to obtain the I$^-$/I$_2$ colorimetric reagent.

**Apparatus:** The spectrophotometer is a BIO-RAD microplate reader (model 3550). The lecture is performed at 490 nm (reference wavelength: 655 nm) after shaking the plate (mix time: 3 sec.). Zero baseline is established with the blank (distilled water). Each value is subtracted with the blank absorbance.

**Urine preparation:** 200 µl of urinary samples are placed into a micro plate. 10 µl of iodine solution is added to each sample. The samples are analysed together with a blank, a negative urine and HES spiked urines.

**HES standard solution:** HES 200/0.5 (Hemohes 6% in 0.9% NaCl) solution was obtained from B/Braun (Switzerland).

**Excretion study:** 100 mg of HES (Haes-Steril, 10% in 0.9% NaCl), obtained from Fresenius Kabi (Norway), was administered to a Caucasian male volunteer aged 30 years old. Urines were collected during 17 days.

**Results and discussion**

After addition of iodine reactive, negative urine specimens remain unchanged and urine samples containing HES instantaneously turned red. Moreover, it was noticed that the intensity of coloration is directly dependent on the HES urinary concentration. The colorimetric method was then validated and evaluated in terms of specificity, precision and reproducibility of the measures, linearity, detection limits and compared to the published GC-MS analysis results.
Precision: Method precision was determined by measuring repeatability and intermediate precision (between day precision) of colorimetric response for two HES spiked urines at 1.2 mg/ml and 600 µg/ml. In order to determine the repeatability of the method, replicate readings (n=6) were carried out on both spiked urine after the addition of the iodine solution. The intermediate precision was evaluated over 3 days by performing six successive readings on the spiked urines, daily. Results show that RSD values are in this case slightly superior than those obtained for repeatability. This can be attributed to the urine spiking but also to the possible variability of UV lamp from a day to another. In Table 1, relative standard deviations (RSD) are listed for repeatability and intermediate precision.

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>HES spiked urine 1.2 mg/ml</th>
<th>HES spiked urine 600 µg/ml</th>
</tr>
</thead>
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<tr>
<td>Day 1</td>
<td>8.96</td>
<td>11.95</td>
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<td>Day 2</td>
<td>7.64</td>
<td>8.44</td>
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<tr>
<td>Day 3</td>
<td>10.89</td>
<td>10.81</td>
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<tr>
<td>Intermediate precision</td>
<td>14.24</td>
<td>15.52</td>
</tr>
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</table>

*Table 1: Precision of the colorimetric method expressed as RSD values (%)*.

Repeatability of urine preparation: The repeatability of urine preparation was evaluated by spiking twenty aliquots of different urine specimens containing 1.2 mg/ml and 600 µg/ml of HES with the iodine solution. The variability of the spiking and the spectrophotometric reading was evaluated by calculating the relative standard deviations (RSD) obtained with the twenty measurements. The RSD's were determined to 9.90% and 15.51%, respectively. It is noteworthy that these values are similar to those obtained for intermediate precision.

*Figure 1: Spectrophotometer response linearity with HES concentration in urine and photo of the corresponding micro plate after the addition of iodine reagent.*
Linearity: Spectrophotometer response linearity was assessed by preparing 14 HES spiked urines covering the concentration range 120-6000 μg/ml. For this range, it can be concluded that the response of the spectrophotometer is proportional to the amount of HES in urine (Figure 1). It can be noticed that for the urinary concentrations superior to 1200 μg/ml, the measured response seems to be saturated.

Kinetic of decolouration: Spectrophotometer response was evaluated in relation to the decolouration of the 14 HES spiked urines covering the concentration range 120-6000 μg/ml over time. A reading of the micro plate was performed each 5 min during 95 min. From this result, it can be said that the decolouration kinetic is quite rapid at the beginning and slows down after 20 min (Figure 2). Nevertheless, the HES spiked urines can be differentiated from the negative urine during 40 min after adding the iodine solution. Practically, the spectrophotometric reading of the micro plate should be done as soon as possible after the addition of the iodine solution to urinary samples.

![Absorbance](image1)

**Figure 2: Decolouration kinetic.**

Limits of detection: The detection limit for the colorimetric method is better than 300 μg/ml of HES in urine as can be seen in Figure 1 and is comparable to the performance obtained by GC-MS. Nevertheless, it can be pointed out that the detection limit can slightly depend on the urinary matrix.

Specificity: The specificity of the colorimetric method was evaluated by performing the spectrophotometric measurements on 10 negative urine specimens from female subjects (F_1-F_10) and 10 negative urine specimens from male subjects (M_1-M_10) after the addition of the iodine solution. In all cases, the measured values were clearly below the values obtained for the HES spiked urines (Figure 3). It can be noticed that numerous urine samples have been simultaneously analysed by GC-MS and the colorimetric method in the laboratory and the specificity of the colorimetric method was evaluated over 1 year.
Application
An excretion study (ES) was analysed simultaneously with the colorimetric and GC-MS methods in order to evaluate the performance of the developed method. For comparison and in order to have an idea of the spectrophotometric response in relation with the HES urinary concentration, HES spiked urines were performed in the same time. Results obtained with the colorimetric method are in accordance with those obtained by GC-MS in terms of HES detection and sensitivity of the method (Table 2 and Figure 4).

<table>
<thead>
<tr>
<th>Code</th>
<th>Period</th>
<th>Volume (ml)</th>
<th>Specific gravity</th>
<th>Concentration* (mg/ml)</th>
<th>Corrected concentration** (mg/ml)</th>
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<tr>
<td>D-12</td>
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<td>250</td>
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<td>0</td>
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<tr>
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<tr>
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<tr>
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<td>1.023</td>
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</table>

Table 2: Data from the excretion study obtained by GC-MS (*concentration of HES estimated from the signal of 2-hydroxyethyl α-glucose, **corrected for specific gravity (1.020)).

![Figure 4: Data from the excretion study obtained with colorimetric detection.](image-url)
Conclusion
The described colorimetric method with iodine reagent was evaluated during 1 year in the laboratory on various urine samples - particularly from endurance sports and especially from samples which were analysed for the presence of recombinant erythropoietin (EPO) - and appears to be a valuable screening method for the detection of HES in urine matrix. This method is simple and reliable for the rapid screening of HES in urine. In our laboratory, the GC-MS method developed by Thevis et al. remains the confirmation method used in the case of a positive response with the colorimetric method.
In the future, it would be necessary to evaluate other HES solutions from various manufacturers, other excretion studies and the stability of HES in urine during several months.

References

Acknowledgement
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