

R.E. Simoni¹⁾, F.B. Scalco¹⁾, L.N.L.F. Gomes²⁾, C.F. Arnaldo¹⁾, T.M. Moutinho¹⁾,
J.F. Paulino¹⁾, S.F. Silva¹⁾, M.L.C. Oliveira¹⁾, F.R. Aquino Neto²⁾

Screening for HES in human urine and possible application for dextran

¹⁾LABEIM, Departamento de Bioquímica, IQ-UFRJ, Rio de Janeiro, Brazil

²⁾LABDOP, LADETEC, IQ-UFRJ, Rio de Janeiro, Brazil

Abstract

This work presents the results and advantages of applying a screening method for hydroxyethyl starch (HES) doping in athletes. The screening is based on Benedict's reaction that detects reducing substances, including HES, dextran and other polysaccharides. A second technique, based on separation by thin-layer chromatography (TLC), was developed to complement the analyses. Screening by Benedict's was applied, during one year, on 4071 athletes and 119 (2.9%) presented results considered suspect. When analyzed by gas chromatography-mass spectrometry (GC-MS) method, no HES positive results were found. The TLC method was applied on 48 out of the suspected 119 urine samples and all were negative for HES, confirming the results obtained by GC-MS. Therefore, it is demonstrated that the TLC procedure may reduce even more the number of urine samples to be analyzed by GC-MS, eliminating in many cases, the necessity of performing this analysis. This simple TLC method can be applied as a preliminary screening for dextran, but its presence in urine needs to be confirmed by other methods.

Introduction

Hydroxyethyl starch consists of D-glucose units joined by α -1-4 linkage, in a branching structure, in which the number of α -1-6 branch points can vary. Hydroxyethyl groups are attached to carbon 2, 3 or 6 of the glucose units (1;2). Hydroxyethyl starch (HES) solutions are artificial colloids that have been officially used as plasma volume expanders (PVE) since 1973 (1;3). In exercise, HES can prevent dehydration, having been generally used to mask blood doping with recombinant erythropoietin (rEPO) (2;3). Its misuse by the athletic community was officially mentioned in 1998 and, since January 2000, the International Olympic Committee (IOC) and more recently, the WADA, prohibit the use of any PVE (4).

The objective of this work was to reduce significantly the number of urine samples that need to be submitted to the usually performed GC-MS methods for detection and identification of HES. A screening method based on Benedict's reaction was developed at LABEIM, a laboratory associated with the WADA accredited Doping Control Laboratory in Rio de Janeiro (LABDOP-LADETEC/IQ-UFRJ), in June 2007, before the PanAmerican Games. Since then, it is routinely being used for the detection of HES in urine of athletes of all kinds of sport competitions. A thin-layer chromatography (TLC) method was adapted for further screening of samples that were considered suspect by the previous Benedict's method (5). Moreover, dextran, which is another polysaccharide detected by Benedict's screening, may also be visualized by TLC.

Materials and Methods

Samples: Urine samples from 4071 athletes participating in different competitions.

Thin Layer Chromatography (TLC): 25 µL of each: urine samples, urine blank, positive urine, dextran solution (20mg/ml), amide solution (4mg/mL) and 50 µL HES solution (0.6 %), all diluted to 250 µL with water, were hydrolysed with 100 µL 3M HCL at 100°C for 60 minutes in a water-bath. A volume of 5 µL from the obtained hydrolysates, together with unhydrolyzed HES and dextran (25 µL each, diluted ten times) and 5 µL glucose solution (1mg/mL) were spotted on a silica gel 60 plate. The developing mixture was: isopropyl alcohol, isoamyl alcohol and ammonium hydroxide (24: 6: 10, v/v/v). After 2 consecutive runs, a 2 mg/mL solution of orcinol in 20 % H₂SO₄, was sprayed onto the dried plate, which was then heated at 180°C for two minutes (6).

Benedict's screening : Urine samples and controls (positive and negative urines, and amide solution), 25 µL each, were diluted ten times with distilled water. Following acid hydrolysis with 100 µL 3M HCl in a water-bath at 100°C for 60 minutes, solutions were neutralized with 115 µL 3M NaOH. One mL Benedict's reagent (5) was added and tubes were placed in boiling water for 8 minutes. After cooling, color and/or precipitates were evaluated. The method, accredited according to ISO/IEC 17025 by the Brazilian Metrological Institute (Inmetro), was validated in terms of specificity, repeatability and detection limit, which was found to be 4.8 mg/mL urine.

Results and Discussion

Concerning the previous Benedict's screening method, urine solutions were evaluated as follows: blue limpid solutions: negative for HES (and sugars); greenish cloudy solutions:

“*suspect +*”; very cloudy greenish or green solutions with yellow or brick precipitate (positive for Benedict): “*suspect ++*” for HES. The Benedict’s method was applied for screening of a total of 4071 urine samples, of which 119 (2.9 %) were considered suspect for HES. These were submitted to GC-MS analysis, but no positive results were found. Out of these 119 suspect samples, 48 were submitted to TLC procedure. HES solution and HES positive excretion urine revealed a characteristic pattern of three bands (a fourth band could be seen when the urine sample was concentrated), owing to glucose and derivatives (Figure 1A); amide solution revealed a strong band at the glucose level; blank urine showed no bands at all and suspect samples showed a main band at the glucose level. None presented the typical HES profile, being therefore, considered negative for HES. These results are in accordance with the results obtained by GC-MS analysis.

The described TLC method, as well as the screening method based on Benedict’s reaction, represent important procedures to limit the number of urine samples that must be submitted to GC-MS methods, which require a high investment, operational and maintenance costs. The procedures are simple, reproducible and low-cost, representing economy of time, equipment, material and qualified staff. The necessary devices for both methods, such as a water-bath, stove or similar are available in any laboratory. The TLC procedure, performed on suspect urine samples, reduces even more the number of samples to be analyzed by GC-MS, eliminating completely, in many cases, the need for this analysis.

It is important to note that dextran and other polysaccharide may also be detected by the described Benedict’s screening method. When submitted to TLC procedure, hydrolysed dextran solutions show a strong band at the glucose level and a weak band near the application spot, which could represent isomaltose or isomaltotriose (Figure 1B). A suspicion of dextran doping may be raised, by evaluation of the chromatographic profiles of unhydrolysed and hydrolysed urine samples. However, the presence of dextran in urine must be confirmed by another method.

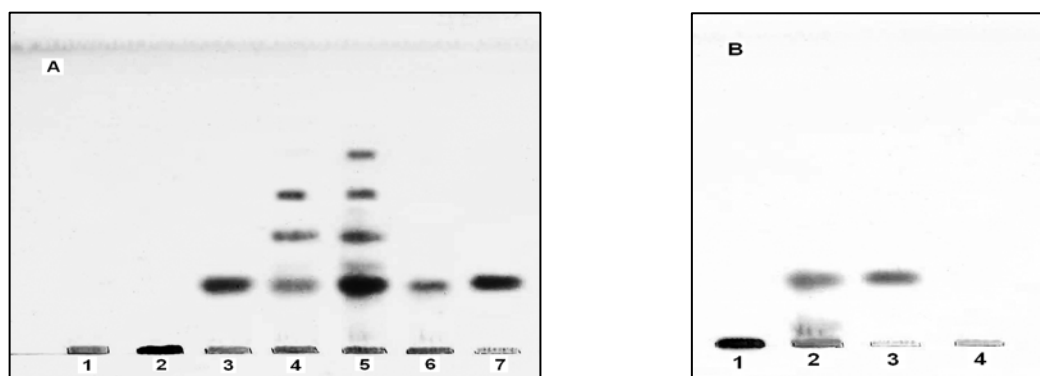


Figure 1- Thin layer chromatogram, showing the characteristic pattern of hydrolysed HES

and dextran A) All lanes, except lane 2, represent hydrolysed samples. Lane 1, urine blank; lane 2, unhydrolysed HES solution; lane 3, amide solution; lane 4, HES solution, lane 5, HES – positive excretion urine; lane 6, urine sample considered “suspect +” by Benedict’s screening method and negative for HES by GC-MS analysis; lane 7, glucose standard. B) All lanes except lane 1 represent hydrolysed samples. Lane 1, unhydrolysed dextran solution; lane 2, dextran solution; lane 3, glucose solution; lane 4, urine blank.

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