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J. Son, D.-H. Kim, M.-J. Kang and M. H. Choi

Screening and confirmation analyses of urinary plasma volume expanders, dextran and HES, in doping control

Doping Control Center, Korea Institute of Science and Technology, Seoul 136-791, Korea

Simultaneous screening analysis of urinary dextran (DEX) and hydroxyethyl starch (HES) was developed based on GC-SIM/MS analysis as their trimethylsilyl derivatives after acid hydrolysis. In screening analysis, α- and β-isomers of glucose and 2-/3-/6-hydroxyethyl-glucose were identified. Samples containing HES residues or samples with urinary concentration of glucose exceeding the concentration of 500 μg/mL of a d_7-glucose spiked urine sample as a quantitative indicator were further analyzed in confirmation process. In confirmation analysis, LC-MS was conducted combined with enzymatic hydrolysis with dextranase and acid hydrolysis for DEX and HES, respectively. After hydrolysis, the urinary extract was derivatized with acetic anhydride and then analyzed by LC-ESI/MS in positive ionization mode. Isomaltose is detected in urine frequently, thus it does not indicate the presence of dextran. For confirmation purpose it is important to quantify the amount of isomaltose, resulting from the enzymatic hydrolysis of dextran.

INTRODUCTION

Urinary plasma volume expanders, dextran (DEX) and hydroxyethyl starch (HES), are used for the treatment of hypovolaemic shocks and prophylaxis of postoperative thrombo-embolic

disorders [1]. In sports its misuse by athletes has been reported. Although chromatography-based mass spectrometric techniques [2,3] give quantitative results in analysis of plasma volume expanders unlike matrix-assisted laser desorption/ionization approach [4], simultaneous identification of DEX and HES could be suggested because an analytical method for each compound is labor-intensive and time-consuming process in screening analysis. Here, we describe rapid and accurate methods linked to GC-MS or LC-MS approaches in both screening and confirmation analyses.

EXPERIMENTS

Hydroxyethyl starch (HETASTARCH; 6% solution in 0.9% NaCl), dextran 1.000, D-(+)-maltose monohydrate, L-(−)-glucose, and dextranase extracted from *Penicillium sp*. were purchased from Sigma. An internal standard, d_7-glucose was obtained from Cambridge Isotope Laboratory.

Sample preparation steps achieved by Thevis *et al*. [2] were introduced. In screening analysis, 20 µL of urine sample hydrolyzed with 3 *M* HCl (200 µL) for 1 hr, at 100 °C including d_7-D-glucose (10 µg) was dried under a nitrogen stream at 50 °C. The dried residue was further dried in a vacuum desiccator over P_2O_5 and KOH for 1 hr and then derivatized with 50 µL of MSTFA/NH_4I/DTE (500:4:2, v/w/w) and 100 µL of ethyl acetate for 30 min at 60 °C. The sample (1 µL) was injected into GC-MSD systems as follow:

Instruments	Agilent 6890 GC & 5973N MSD
Column	Ultra-2 (25 m length × 0.2 mm I.D. × 0.33 µm thickness)
Injection parameter	Injection mode: Split (10:1) Initial temperature: 280 ºC
Flow rate program	Initial flow: 0.8 mL/min (Helium) with constant flow
Oven program	Initial temperature: 150 ºC Rate: 15 ºC/min Final temperature: 300 ºC Final time: 6 min
MS parameter	Ionization mode: EI at 70 eV Acquisition mode: SIM (191, 192, 204, 206, 217, 220, 235, 248, 261) Interface temperature: 300 ºC Source temperature: 230 ºC Dwell time: 100 msec

In confirmation of DEX, 20 µL of urine pre-washed with diethyl ether was hydrolyzed with 0.1 *M* citrate buffer (pH 6.0, 40 µL) and dextranase solution (1.5 mg/mL water, 5 µL) for 1 hr at 37 °C [3]. For HES, urine was hydrolyzed with 3 *M* HCl. In both cases, hydrolyzed solution was evaporated and dried in a vacuum desiccator and then derivatized with 310 µL of pyridine/acetonitrile/acetic anhydride (1:15:15, v/v/v) for 30 min at 40 °C. Final solution was mixed with ethyl acetate (2 mL) and water (2 mL), and organic extract was evaporated and reconstituted with 10% acetonitrile (100 µL) for being injected into LC-MS system.

Instruments	Agilent 1100 series HPLC & Agilent XCT plus ion trap MS
Column	Agilent Zorbax Eclipse XDB-C18 (100 × 2.1 mm I.D., 3.5 µm)
Pumping system	Flow rate: 0.2 mL/min Mobile phase: (A) 0.1% CH_3COOH in 5% acetonitrile (B) 0.06% CH_3COOH in 95% acetonitrile Time program: 0 min (5%, B), 5.0 min (5%, B) 10 min (95%, B), 15 min (95%, B) 15.1 min (5%, B), 23.0 min (stop, B)
Mass parameter	Electrospray source: 35 psi nebulizer gas Dry temperature: 350 ºC Polarity: Positive Scan range: *m/z* 50 – 1.000 MRM (mass/width): 452/1.0, 457/1.0, and 473/1.0

RESULTS & DISCUSSION

Acid hydrolysis of DEX and HES was conducted for rapid screening analysis and resulted in 2 isomers of glucose and 4 main analogues of hydroxyethyl-glucose derived from DEX and HES, respectively. Two characteristic ions for each compound are monitored by GC-MS in selected-ion monitoring mode (*Fig*. 1). Acetylation in confirmation analysis was conducted to improve chromatographic properties of hydrophilic saccharides in urinary matrix and it was found to be most efficient for the separations at a desirable sensitivity level. Acetylated isomaltose and hydroxyethyl-glucose produced intense ions corresponding molecular ion, and significant fragments, *m/z* 641, 581, 521 for *iso*-maltose and *m/z* 397, 337, 277 for hydroxyethyl glucose, resulting from consecutive losses of acetic acid (− 60 Da) in MRM analysis.

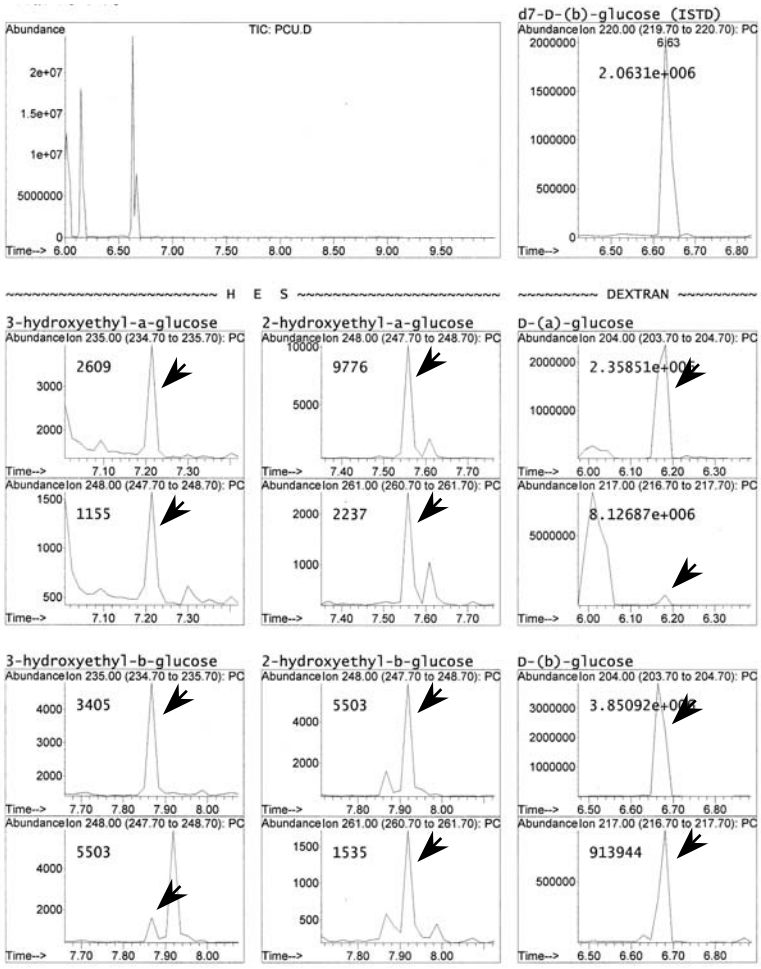

***Fig*. 1.** Screening window obtained from the positive control urine spiked with hydroxyethyl starch (60 µg/mL) and dextran 1.000 (1,000 µg/mL).

However, detection of urinary glucose as an indication for an administration of DEX can be achieved only when there is a cut-off value against naturally occurring amounts between individuals or identification of its origin using isotopic discrimination.

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