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Quantitative analysis of glycerol levels in human urine by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Glycerol has the latent capacity to act as a plasma volume expander and disguise blood doping practices. Therefore, it has been prohibited in sports as a masking agent by the World Anti-Doping Agency (WADA) since January 2010 and a urinary threshold (1 mg/mL) was recommended recently [1]. The purpose of this study was to establish and validate a novel quantitative method for the determination of urinary glycerol concentrations using a liquid chromatography–tandem mass spectrometry approach. This simple yet highly specific method made use of the derivatization of glycerol by benzoyl chloride in aqueous solution at 40 °C followed by analysis via LC–ESI-MS/MS without sample pre-concentration or cleanup. The assay was linear over the concentration range of 1.0–1000 μg/mL for glycerol in human urine. The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were 0.3 μg/mL and 1.0 μg/mL, respectively. The intra- and inter-day precision of the method at three concentration levels (3, 500 and 900 μg/mL) was less than 12.2%. The method also afforded satisfactory results in terms of accuracy, derivatization yield, extraction recovery, matrix effect and specificity. The method has been successfully applied to the detection of glycerol in "Quality Assurance Program" samples provided by the World Association of Anti-Doping Scientists (WAADS) and routine doping-control samples in our laboratory.

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1. Introduction

Glycerol (1,2,3-propanetriol) is a naturally occurring compound that constitutes the backbone of the triglyceride molecule. Normal plasma physiological concentrations of glycerol in adult humans are between 4.6 and 27.6 μg/mL [2]. The analysis of a large number of routine doping-control urine samples shows that the endogenous concentration of glycerol is usually less than 20 μg/mL and does not exceed 140 μg/mL [3].

The ingestion of glycerol hyperhydrates the body, increasing the volume of water above the normal level, which can play a role in lowering hemoglobin concentrations. Therefore, the potential of glycerol to act as a plasma volume expanding agent makes it attractive to athletes for masking possible blood doping practices [4]. As a consequence, glycerol was added to the WADA prohibited list in January 2010 and all forms of glycerol administration are prohibited in sports [1].

Traditionally, the analysis of glycerol has been performed using enzymatic spectrophotometry [5,6], high-performance liquid chromatography [7,8] or gas chromatography–mass spectrometry [3,9–11]. The enzymatic methods are convenient but susceptible to interference [6]. Rosenberger et al. [7] developed a method to quantify free glycerol and *myo*-inositol from plasma and tissue by high-performance liquid chromatography after sample derivatization, but the limit of quantitation (LOQ) of glycerol was 230 μg/mL in plasma and 589 μg/g in tissue.

The GC–MS methods require extensive sample pretreatment and derivatization before injection into the GC–MS system. Thevis and co-workers [3] reported a GC/isotope-dilution MS approach to quantifying urinary glycerol concentrations for the purposes of doping-control. Nevertheless, the overnight sample drying process employed adds considerable complexity to the method and is also time-consuming and expensive, which reduces the efficiency of high throughput screening analysis. In addition, the linearity range for the measurement of glycerol in human urine was normally lower than 100 μg/mL.

Another article [12] described a liquid chromatography-single quadruple mass spectrometry process (LC–MS, SIM mode) for directly measuring human serum glycerol levels using $[M+Na]^+$ as an ion for quantitation. The formation of the sodium adduct in the

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spray chamber of the mass spectrometer relies on the presence of trace amounts of sodium ion in the mobile phase, presumably from the glass solvent containers. The amount of sodium ion in the system is not standardized, so that quantitative results are not reproducible.

The work presented here is aimed at the development and validation of a novel analytical method for the determination of urinary glycerol levels using LC–ESI-MS/MS technique with simple sample preparation. The urine sample was first derivatized in aqueous solution under mild conditions, separated by HPLC then analyzed by MS/MS technique. In doping-control laboratories, this method would allow the analysis of large amounts of samples in shorter time and lower cost.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, *n*-hexane, glycerol, d_5-glycerol (internal standard, 98%), benzoyl chloride and glyceryl tribenzoate of HPLC grade, were obtained from Sigma-Aldrich (St. Louis, USA). Ammonium formate and formic acid of HPLC grade were purchased from Fluka (Pittsburgh, USA) and DikmaPure (Lake Forest, USA), respectively. Benzene-1,3,5-triyl tribenzoate of analytical grade was a kind gift from Pharmaceutical School, Beijing University. Deionized water was purified with a Milli-Q Academic ultra-pure water system (Millipore, Milford, USA). All other chemicals were analytical grade and used as received.

2.2. Chromatographic and mass spectrometric conditions

Chromatography was performed on an Agilent 1200 series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). Separation was achieved on an Eclipse XDB-C18 column (2.1 mm × 100 mm, 3.5 μm, Agilent Technologies Inc.). The mobile phase was composed of 10 mM aqueous ammonium formate buffer (which was adjusted to pH 3.5 with formic acid) (eluent A) together with acetonitrile (eluent B). A gradient was employed starting at 60% B and increasing to 90% B within 10 min, and re-equilibrated at 60% B for 4 min. The flow rate was set at 0.4 mL/min, the column oven temperature at 40 °C and an injection volume of 20 μL.

Mass spectrometric detection was carried out using an Agilent triple-quadruple 6410B mass spectrometer (Agilent Technologies Inc.) equipped with an electrospray ionization (ESI) source. Multiple reaction monitoring (MRM) mode was used to detect the analytes in positive ionization mode. The spray voltage was set at 4000 V and the ion source was operated at 330 °C. Nitrogen was used as the nebulizing and the drying gas, and the pressure was set at 40 psi.

2.3. Preparation of calibration standards and quality control (QC) samples

A stock solution of glycerol was prepared in deionized water at a concentration of 10 mg/mL. Standard solutions (10, 100, 250, 500, 750, and 1000 μg/mL) were prepared by serial dilution of the stock solution with 4 N aqueous sodium hydroxide. Low, medium and high concentration quality control (QC) samples (3, 500, and 900 μg/mL) were prepared in a similar way. The stock solution of internal standard (IS), d_5-glycerol (10 mg/mL) was prepared in deionized water and diluted with 4 N aqueous sodium hydroxide to a final concentration of 500 μg/mL. The stock solution for another internal standard of benzene-1,3,5-triyl tribenzoate (10 mg/mL) was prepared in methanol and diluted with *n*-hexane to a final concentration of 500 μg/mL. All solutions were stored at 4 °C and used within one month after preparation.

2.4. Sample derivatization

In a glass screw-top 10 mL test tube, 850 μL of 4 N aqueous sodium hydroxide, 100 μL of a urine sample, 50 μL of IS solution (500 μg/mL) were combined. To start the reaction, 0.5 mL of *n*-hexane and 100 μL benzoyl chloride were added to the above sample solution and capped with a Teflon-lined closure. The solution was mixed gently then incubated in a metabolic incubator (Julabo, SW22, Germany) at 90 rpm and at 40 °C for 4 h. Following incubation, 1 mL of deionized water was added to the reaction mixture and benzoyl ester derivatives were extracted with 5.0 mL of *n*-hexane. After centrifugation at 2500 rpm for 2 min, 100 μL of the supernatant was transferred to a 2 mL autosampler vial containing 900 μL of *n*-hexane. A 10 μL portion of this solution was injected into the LC–MS/MS system for analysis.

Calibration standards and quality control samples were prepared in a similar way: in a glass screw-top 10 mL test tube, 750 μL of 4 N aqueous sodium hydroxide, 100 μL of standard solutions, 50 μL of IS solution (500 μg/mL), 100 μL of blank urine were added. Then the derivatization and extraction steps were performed by the procedure discussed above.

2.5. Method validation

The qualitative and quantitative determination of glycerol in human urine was validated for linearity, lower limit of detection, lower limit of quantitation, intra- and inter-day precision, accuracy, derivatization yield, extraction recovery, matrix effect and specificity. The procedures for validation are discussed below.

2.5.1. Linearity

Three independent calibration curves and three replicates of each QC sample (3, 500, and 900 μg/mL) were prepared in blank urine with no detectable amount of glycerol and measured on three different days using ten calibration points from 1.0 to 1000 μg/mL. Calibration curves were plotted as the peak area ratio of analyte to the internal standard versus concentrations. Linearity was considered acceptable with correlation coefficient of ≥0.999.

2.5.2. Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ)

The LLOD was defined as the lowest concentration that can be detected with a signal-to-noise ratio ≥3. The LLOQ was defined as the minimum content at which the analyte could be quantified with satisfactory accuracy and precision (RSD < 20%).

2.5.3. Intra-day precision

The intra-day precision of the method was assessed by performing replicate analyses of QC samples assayed with a set of calibration standards. Within one day, ten urine samples spiked with low, medium and high concentrations (3, 500, and 900 μg/mL) of glycerol were prepared and analyzed. The intra-day precision was calculated for each concentration level.

2.5.4. Inter-day precision

The inter-day precision was based on the assay of replicate analyses of QC samples run with a set of calibration standards. On three consecutive days, a total of ninety urine samples of low, medium and high concentrations (3, 500, and 900 μg/mL) of glycerol were prepared and analyzed and the inter-day precision was calculated for each concentration level.

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