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High-throughput screening method for glycerol in urine using a "dilute-and-shoot" liquid chromatography-tandem mass spectrometry approach

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Abstract

Glycerol is listed under "S5 Diuretics and other masking agents" on WADA's Prohibited List (WADA 2014). The possible doping effect of glycerol is related to hyperhydration and manipulation of blood parameters. An intake of 1 g glycerol/kg body weight in combination with large amounts of fluid (10-25 mL/kg body weight) is recommended. The resulting hyperhydration can be of benefit for endurance sport, especially under heat conditions and situation when fluid intake is limited. The hyperhydration can also influence the blood parameters of the Biological Passport and can therefore be highly relevant in connection with blood doping. In a study by Koehler *et al.* 2011 a significant reduction of hemoglobin and hematocrit could be observed in a cross-over study for a group of cyclists at rest. But this effect is controversially discussed in the literature, because another study demonstrated that the effect on the blood parameters is negligible under endurance exercise (Koehler *et al.* 2014).

Low concentrations of glycerol are detectable in the majority of urine samples because of the metabolism of triglycerides and exogenous intake by food and pharmaceuticals. Two studies, one by Thevis *et al.* 2008 and one by Kelly *et al.* 2013, observed concentrations of glycerol below 20 μ g/mL urine for the majority of athlete samples. Concentration levels up to several hundred μ g/mL were detectable in only a small number of samples. The threshold value introduced by WADA which was in force in april 2014 is 1 mg glycerol/mL urine (WADA 2013). An intake of glycerol with the intention of doping in the recommended dosage leads to concentrations of several mg/mL urine.

Quantification of glycerol in urine is traditionally based on derivatization with subsequent detection by gas chromatography coupled to mass spectrometry (GC-MS). A new screening method for glycerol using a "dilute-and-shoot" approach and detection by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was developed. After dilution and addition of the internal standard the sample is directly injected on the LC-MS/MS system. Advantage in comparison to the traditional method based on detection by GC-MS is a clearly faster sample preperation.

Introduction

Glycerol as doping agent is discussed in the relation of hyperhydration and manipulation of blood parameters. The effect of hyperhydration after the recommended intake of 1 g glycerol/kg body weight in combination with large amounts of fluid (10-25 mL/kg body weight) can be of advantage in endurance sports. As aresult of hyperhydration a manipulation of blood parameters of the Biological Passport, especially hemoglobin and hematocrit, is thinkable. Because of the naturally occurring concentrations of glycerol a threshold level was introduced by WADA (WADA 2013). Gas chromatography coupled to mass spectrometry (GC-MS) after derivatization is the traditionally used method for the detection of glycerol in urine (Thevis *et al.* 2008, Kelly *et al.* 2013). The presented method uses high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) for detection and the "dilute and shoot" approach for sample preparation.



Experimental

Chemicals

Solvents and reagents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), and Macherey-Nagel (Düren, Germany). Water was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany). Glycerol and glycerol- ${}^{13}C_3$ were obtained from Sigma-Aldrich.

Analysis of urine samples by HPLC-MS/MS

For the determination of glycerol in urine using HPLC-MS/MS 200 μ L urine were diluted with 800 μ L internal standard solution in acetonitril/water (75/25, v/v) containing glycerol-¹³C₃ (c = 0.625mg/mL). Analysis was carried out on an Agilent 1290 series HPLC (pump G4110, autosampler GU226A, and column oven G1316C) coupled to a G6490A Triple Quad LC-MS (Agilent Technologies, Waldbronn, Germany). For chromatographic separation a Hypersil Gold Amino column (150 x 2.1 mm, 3 μ m) (Thermo Fisher Scientific, Bremen, Germany) was used. Column temperature was set to 30°C. The chromatographic separation was carried out using a binary gradient of 1mM ammonium acetate and 0.1% acetic acid in water (A) and acetonitrile (B) with a flow rate of 400 μ L/min and a gradient of 80% B (0 min), 80% B (1.5 min), 10% B (1.6 min) and 10% B (3 min). Injection volume was set to 5 μ L. For mass spectrometry negative ion mode and multiple reaction monitoring (MRM) were used. The following transitions (collision energy (CE) in brackets) were used: glycerol 151 \rightarrow 59 (CE 10) and glycerol-¹³C₃ 154 \rightarrow 59 (CE 10). The capillary voltage was set at -3000 V and the nozzle voltage at -1000 V. The gas temperature was adjusted at 300°C and the sheat temperature at 400°C. Nitrogen was used as drying gas (gas flow 14 L/min and sheat gas flow 11 L/min) and as collision gas. Concentrations were calculated by using a calibration curve ranging from 0.5 to 2.5 mg/mL. Urine samples exceeding the range of the calibration curve were appropriately diluted and reanalyzed.

Analysis of urine samples by GC-MS

For sample preparation 50 μ L urine sample and 100 μ L internal standard solution containing glycerol-¹³C₃ in methanol (c = 0,5 mg/mL) were evaporated to dryness and afterwards derivatized with 25 μ L pyridine and 75 μ L MSTFA-ethanethiol-NH₄I (1000:2:6, v/w/v) for 20 min at 70°C. Analysis was carried out using a HP6890 gas chromatograph coupled to a HP5973 mass specific detector and a HP7683 autosampler (Agilent). For chromatographic separation an Ultra column (17 m × 0.2 mm × 0.11 μ m, Agilent) was used. Carrier gas was helium at a flow of 0.6 mL/min. 0.2 μ L were injected using spilt injection (1:100) at 280°C. The mass spectrometer was operated in the electron impact mode (EI) at 70eV. The following temperature program was used: 0-1 min isothermal at 80°C, 25°C/min to 140°C and 60°C/min to 300°C where it was held for 2 min. Mass spectra were acquired in selected ion monitoring (SIM) mode with a dwell time of 20 msec for each ion (m/z 103, 117, 205, 218, 221, 293). Quantification was carried out as described before.

Results and Discussion

The traditionally used screening methods for the detection of glycerol in urine base on the detection by GC-MS (Thevis et al. 2008, Kelly et al. 2013). Therefore the urine samples are evaporated to dryness and afterwards derivatizated. Because of these two necessary, but time-consuming sample preparation steps an alternative screening method using LC-MS/MS was developed. Therefore a mass transition is required which is suitable for the detection of glycerol. Glycerol ionizes in the positive mode as adduct ion with sodium and ammonium, a protonated molecular ion can not be detected. The disadvantage of the adduct ion with sodium is the absence of suitable product ions in the MS/MS mode. The appearance of adduct ion with ammonium is limited to some instrument types. In the negative mode an adduct ion with the acetate can be observed if a buffer with ammonium acetate and acetic acid as mobile phase is used. In the MS/MS mode a suitable product ion in the form of the acetate ion can be detected and the transition m/z 151 [M+Ac] \rightarrow 59 [Ac] results. The advantage of this transition is that the application is independent of the instrument type. In order to obtain a simple sample preparation the "dilute and shoot"-approach was chosen. An attempt using reversed chromatography for separation of glycerol shows clearly the poor retention of glycerol on this type of separation column. In addition the peak shape of glycerol is also poor because of tailing. An alternative for the chromatographic separation of polar compounds offers the separation in normal phase (NP) mode. In comparison to the separation in the reversed phase mode an improved retention of glycerol by using an amino column as stationary phase was obtained. A comparison between a blank urine and a control sample with 1 mg glycerol/ml shows no peaks, which interfere with glycerol as analyte and its isotopically labeled internal standard. One interfering substances which is contained in every urine elutes well separated from the glycerol as analyte.





Figure 1: NP-HPLC-MS/MS chromatogram of a blank urine sample and a control urine sample containing 1 mg/mL glycerol. (A) Shows glycerol with MRM m/z 151 \rightarrow 59, (B) glycerol-¹³C₃ with MRM m/z 154 \rightarrow 59.

The validation of the method confirms the suitability as screening method in doping analysis. Intra-assay precision is 1.0% for the measurement over three days. The inter-assay accuracy is determined to be 99.2%. These results are comparable to these which are obtained for the reference method on the GC-MS.

The investigation of excretion urines underlines also the suitability of this method. After intake of 1 g glycerol/kg body weight in combination with 10 mL water/kg body weight the highest concentrations of glycerol (16.7 mg/mL) can be detected after 3 hours. After 8 hours glycerol is eliminated nearly completely and glycerol concentration reaches the natural excretion levels.

In addition 327 doping control routine samples were analyzed using this HPLC-MS/MS method and in addition using GC-MS. In three samples concentrations above 0.1 mg/mL could be detected. The quantification results for these three samples are comparable between HPLC-MS/MS (0.488, 0.435 and 0.728 mg/mL) and GC-MS (0.469, 0.441 and 0.724 mg/mL).

Conclusions

This newly developed HPLC-MS/MS method for the detection of glycerol in urine offers an alternative to the traditionally used detection by GC-MS after derivatization. Advantages are the shorter analysis time because of the used "dilute and shoot"-approach for sample preparation. Thereby the sample preparation is minimized to a minimum. In addition to the successful validation the investigation of excretion samples and doping control routine samples confirms the suitability of the method.

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Lecture



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