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Determine A Glycerol Cutoff Value Among Jordanian Athletes And The Effect Of Use Hubbly Bubbly On Urinary Glycerol Threshold From Anti Doping Perspective

Shrouq Mahameed [1*] and Kamal Hadidi [2]

[2]Department of Pathology and Microbiology and Forensic Medicine, Faculty of Medicine, [1]The University of Jordan, Amman, Jordan.

[*]Corresponding author
e.mail: shrooq_mhameed@hotmail.com

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Abstract

Problems: Since 2010 World Anti-Doping Agency (WADA) put glycerol in their prohibited substances list considering it is masking ability. Exogenous glycerol elevates plasma glycerol concentrations up to a point where glycerol begins to appear significantly in urine. Smoking hubbly bubbly that contained glycerol as humectant can affect the urinary glycerol threshold especially when a 41.3% of the Jordanian universities students smoke hubbly bubbly. The aim of the study is to assess the normal urinary glycerol range among the Jordanian athletes and determine hubbly bubbly influence on this range.

Experimental approach: Urine samples were collected from Jordanian athlete & non-athlete volunteers. In addition to urine sample from 20 Adult healthy Jordanian athletes who smoked hubbly bubbly. All samples were analyzed by GC/MS.

Findings: Analysis showed that 80% of the normal urinary glycerol concentration was ≤ 20µg/ml.

Conclusion: Hubbly bubbly regime used in Jordan showed no effect on urinary glycerol concentration.

Key words Hubbly bubbly, Glycerol, GC/MS, athlete, Sport doping.

Abbreviation: GC/MS: gas chromatography/mass spectrometer, QC: quality control, I.S: internal standard, TMS: trimethylsilyl, HB: Hubbly bubbly

Introduction

Glycerol $C_3H_8O_3$ is a neutral, hygroscopic, colorless, clear liquid with a molecular mass of 92.0 grams per mole. Glycerol the trihydroxy sugar alcohol is the backbone of the triglyceride and an intermediate in carbohydrate and lipid metabolism. It is normally found in the blood as a product of the lipolysis with normal serum concentrations of glycerol at rest equal to 4.6 µg/ml and can increase up to 27.6 µg/ml during high lipolysis rate that is associated with prolonged exercise or caloric restriction [(1)]. Once exogenous glycerol is introduced to the body either by infusion or ingestion it reached a point where started to appear significantly in the urine [(2)]. Glycerol ingestion with the addition of fluid has been used to increase total body water in order to improve thermoregulation and endurance during exercise in hot environments [(3)]. These findings have promoted glycerol doping for it is hyperhydration effect in order to make up the negative effects happened with dehydration. Dehydration during exercise is known to decrease intracellular, interstitial and plasma volumes with a reduction in stroke volume, increasement in heart rate which is led eventually to reduce the exercise performance [(4)]. Additional fluid loss during exercise with a reduction in sweat glands` output disturbed the evaporative cooling system in the body and cause body temperature elevation. Increased blood volume and total body weight by an osmotic agent ingestion showed regulation effects on the core temperature, stroke volume, heart rate response and maintained the athletic performance as a consequence [(1)]. Glycerol existence in the blood stream created an osmotic gradient that promoted water absorption from the tissue leading to plasma volume enlargement. With this ability to increased plasma volume, the possibility to mask doping practice became higher. Glycerol as a consequence has been

reported to be a prohibited substance since 2010 by the WADA and recognized as a masking agent in sports and the athlete forbidden from participating in competition if urinary glycerol concentration exceeds 1 mg/ml [5]. Glycerol is widely used in food, cosmetic and pharmaceutical industries; it can contribute in these products as a plasticizer, flavoring, denaturant, emollient, antimicrobial, thickener and solvent. Hygroscopicity (humectancy) is one of the most valuable properties of glycerol that promoted use it in many applications to give products the desired consistency of softness, flexibility, creaminess, and shelf life. One of these products is mua'sel which used in the hubbly bubbly. Hubbly bubbly which is born in India in the 1400s and known by other names like a hookah, narghile, water pipe, goza and shisha was quickly spread to countries in the Middle East [6]. Recent evidence showed that a 41.3% of the Jordanian universities students used hubbly bubbly at least one time monthly [7]. Hubbly bubbly tobacco mixture which locally known as mua'sel consists of one or more types of virgin tobacco (Nicotina tobaccum) mixed with saccharides as a sugar molasses or honey and glycerol [8]. The amount of additives added in mua'sel preparation for a kilogram of tobacco are 250-350ml glycerol, 125-250 honey, and 50ml flavor solution; which were mixed together with 1 kg unflavored tobacco and left for 3 weeks before being used [9]. With this high amount of glycerol the potential to cause elevation of urinary glycerol concentration can appeared after smoking hubbly bubbly. Especially, when pyrolysis studies on glycerol samples showed no combustion and as a result glycerol transferred intact in the smoke [10]. So, elevated glycerol level can affect the urinary glycerol base line in the athletes and lead WADA to prevent the athletes from participating in competitions.

Materials and Methods

Chemicals

Glycerol ≥ 99.5%, 1, 2, 4 butantriol 98% as internal standard, anhydrous sodium sulfate powder ACS reagent, ≥99.0% and N, O bis (Trimethylsilyl) trifluoroacetamide (BSTFA) Trimethylchlorosilane (TMCS) 99:1 were purchased from Sigma-Aldrich®.

Volunteers

Non athlete volunteers were from the Jordan university community, where the athlete

volunteers were from the physical education faculty. Informed consent was taken from the participants after explaining the study to them. Volunteers were divided into 50 Jordanian non athlete volunteers and 50 Jordanian athletes who participated in the determination the normal glycerol urinary concentration. Another 20 athletes were engaged in the hubbly bubbly smoking part of the study. Athlete group includes 28 male and 22 female with the following characteristics: age between 18-25 years, weight: 73.3 ± 7.6 kg. Where a non athlete group is included 27 male and 23 female with the following characteristics: age between 18-25 years, weight: 70 ± 4.1 kg. The inclusion criteria were males and females who their age between (18-25) years, not taking any medication during the study and no history of chronic disease, especially kidney or liver disease, diabetic 1 and 2 or lipid metabolism related illness. Where is the exclusion criteria were volunteers with diabetic mellitus disease type 1 or 2, lipid metabolism related illness and volunteers who take an energy drink frequently. Urine sample is obtained from the participant using sterile midstream catch after washing hands to avoid cross contamination if the participants used glycerol containing products [11].

Hubbly bubbly smoking setting

The 20 athletes who participated in the hubbly bubbly smoking part of the study were interviewed to identify their smoking habits. Before the hubbly bubbly smoking part began baseline urine sample was taken. Volunteers were given a hubbly bubbly to smoke with 20 grams of flavored mua'sel head and were allowed to smoke as their habit for 45 min. The same brand and flavored mua'sel were used for all the participating athletes. The used hubbly bubbly has a metal stem of 37cm long and 1 cm internal diameter, bowl has a capacity of 400 ml and the hose have 150 cm length and 1.0 cm internal diameter. Volunteers regimes to smoke hubbly bubbly were studied individually (Puffs numbers per a minute, puffs per a head and retention time for puff holding). Each individual took 3 hubbly bubbly and between each session participants asked to gave a urine sample to assess the effect of hubbly bubbly on the urinary glycerol concentration.

Sample preparation

Sample preparation started by centrifuge the sample at 4000 rpm for 5 min in order to

remove any impurities or debris in urine. Then 20 µl of urine was placed in a glass reagent tube with 20 µl of the I.S and dried in a vaccum dessiccator over a sodium sulfate anhydrous powder [12]. Then the dry residue was dissolved with 100µL of BSTFA–TMCS and heated for 30 min at 80°C. After cooling at ambient temperature, the samples transferred the sample to the GC-MS vials to be injected in the GC/MS.

GC/MS analysis was conducted in the toxicology laboratory at the Jordan University hospital using GC- 2010 Shimadzu with a DB-5MS column (inner diameter 0.25 mm, film thickness 1.0 µm, length 30 m). Temperature program started at 100°C which is held for 10 minutes then increased by 8 ºC/min to 290°C, entire programmed run completed in 23 min. Helium was used as carrier gas with a flow rate 1.3 ml/ min, pressure 103.1 kpa and linear velocity at 43.1 m/s. The injector temperature was set to 280°C, the interface temperature to 280°C and the ion source temperature to 230 ºC. One µl of the derivatized sample were injected in splitless mode with electron ionization (EI) at 70 eV using scan mood m/z ratio 50-600with with a scan speed equal to 1250. Selected ions considered for glycerol qualitative analyses according to WADA guidelines: m/z103, 218 and 205 and the quantifier ion were m/z 218 [12,13].

Method validation

Method validation parameters which considered in the current study attempted to harmonize between the food and drug administration (FDA) guidelines and the European Medicine Agency (EMA) guidelines [14,15]. The parameters were linearity, specificity, the lower limit of detection (LOD), the lower limit of quantification (LLOQ), precision, accuracy, and matrix effect.

Linearity

Calibration curve of glycerol prepared with 11 different concentrations (2.5, 5, 10, 25, 50, 75, 100, 125, 150, 200 and 250) µg/ml spiked in pooled urine sample.

Accuracy

To evaluate the accuracy, six replicated samples at three different QC levels (5, 50 and 125) µg/ml concentrations were prepared by spiking the blank pooled urine sample with glycerol.

Precision

Six replications of the three different QC level (low, medium and high) concentration

were prepared by spiking the blank pooled urine sample with (5, 50 and 125) µg/ml and analyzed within one day for Intraday precision and over three successive days for Interday precision.

The LOD and the LLOQ

The LOD and LOQ was assessed depending on the standard deviation of the response and the Slope of a special calibration curve constructed from 4 low concentrations (1, 2, 2.5 and 5) µg/ml glycerol spiked in pooled urine sample.

Specificity

The specificity of the method was determined by obtaining positive results through compare between a known reference material of glycerol spiked in urine samples, coupled with negative results from samples which are did not contain the analyte.

Result and Discussion

Method validation

Linearity test results showed high linear relationship with a high R^2 value equal to 0.997 for urinary glycerol calibration curve. Accuracy results obtained from spiked urine samples at three different QC level (5, 50 and 125) µg/ml concentrations, gave results within (98%-102.6%), these results agreed with FDA criteria [14]. On the other hand assessment of precision at three different QC level (5, 50 and 125) µg/ml concentrations was performed intraday and yielded CV% values (0.16%, 2.8% and 4.9%). Where is interday precision having (6.1%, 1.8% and 0.4%) CV% values. The LOD result was 0.536µg/ml and 1.625µg/ml LLOQ. These results adapted for glycerol with respect to the normal urinary glycerol concentration range studied in the current study. Specificity confirmed with respect to the retention time of urinary glycerol at~ 11.34 min and the fragment ions which are uniquely used for glycerol identification like m/z 218, m/z 205, and m/z 103 [12,13].

Sample analysis

Glycerol- TMS eluted at ~11.34 min and I.S- TMS eluted at ~12.77 min using the current method (Figure 1). Urine sample GC/MS analysis showed normal urinary glycerol concentrations as in (Figure 2a). Different sport categories were involved in this study like endurance sport, strength sport and competition sport. The distribution of

normal urinary glycerol concentration with respect to sport disciplines demonstrated in (Figure 2b). Hubbly bubbly effect on the urinary glycerol concentration after each session for the 20 athletes demonstrated in the (figure 2c).

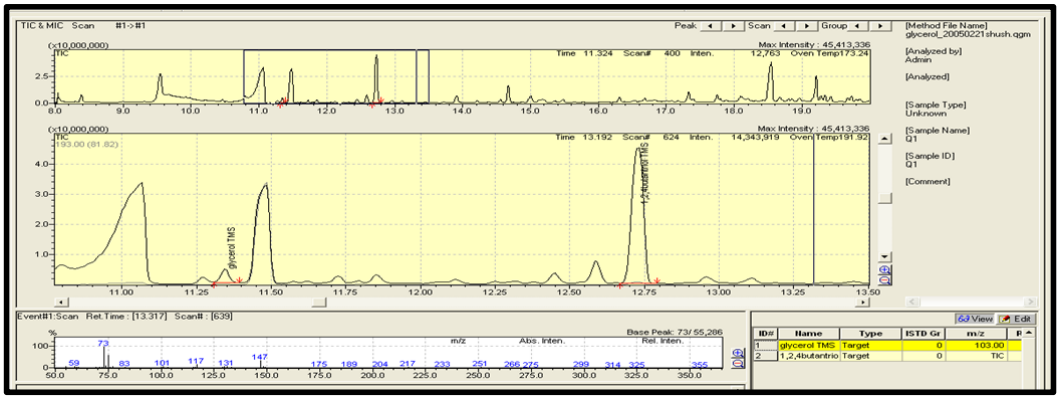

Figure 1. Chromatogram of glycerol- TMS and I.S- TMS peaks

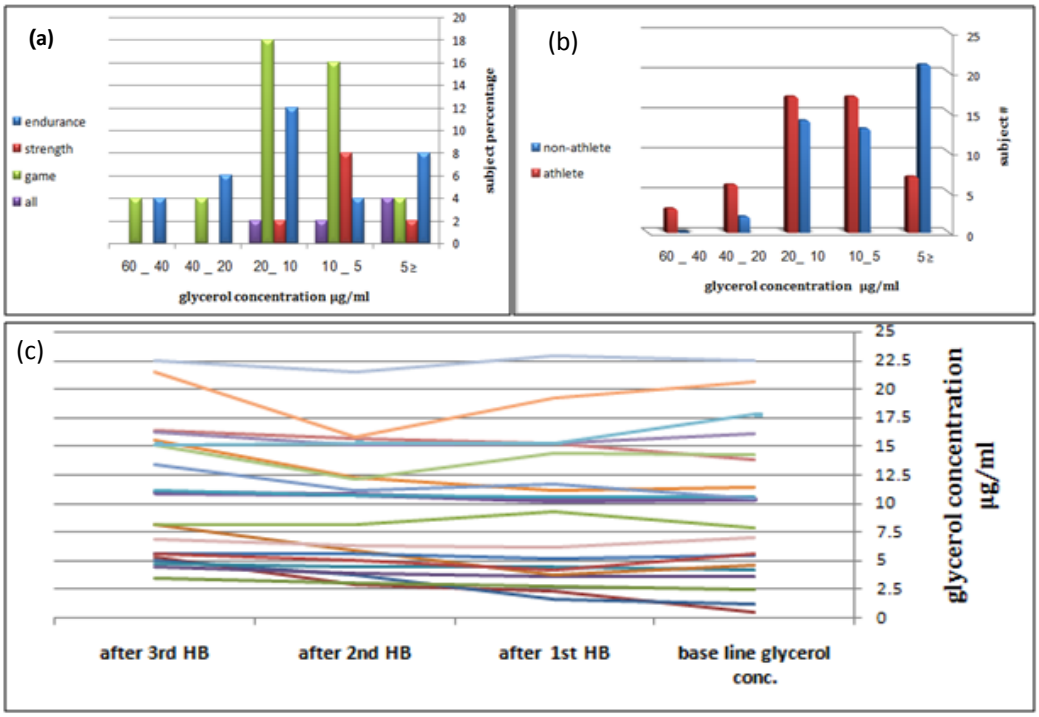

Figure 2a. Urinary glycerol concentration in the two target populations, Figure 2b. Relationship between the sport kind and the urinary glycerol concentration, Figure 2c.

In principle, highly inert columns coated with a polar stationary phase can be used to analyze glycerol without derivatization but the column inertness cannot be maintained in

routine analysis. So, trimethylsilylation of the free hydroxyl groups in glycerol ensured excellent peak shapes, good recoveries, low detection limits and enormously improved the ruggedness of the procedure [16]. Glycerol analysis can be affected by Urea normally presence in urine which can mask or caused loss of glycerol- TMS peak resolution (Figure 3). One minute holding temperature program at 100ºC at the beginning of the oven temperature as in original method by Iga and his colleagues [13] can show this phenomena, but modification on the holding time program by shifting it from 1 min to 10 min can enhance the resolution between the glycerol- TMS peak and the urea peak as in (Figure 1).

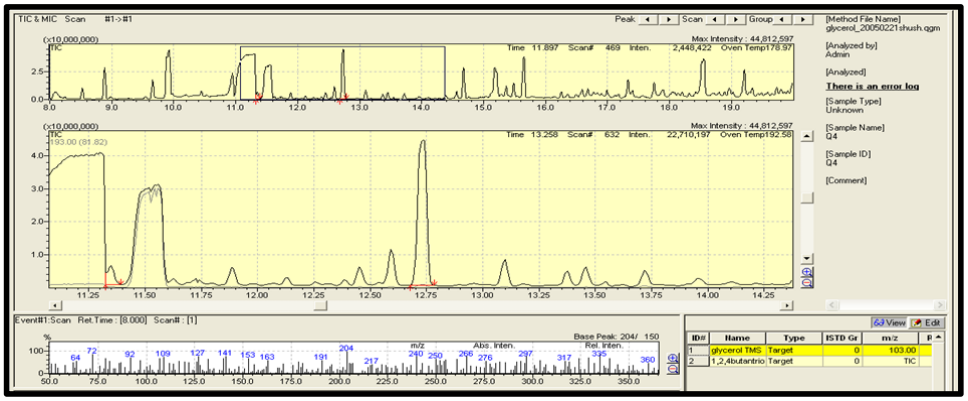

Figure 3. loss of resolution between the urea peak and the glycerol-TMS peak

Glycerol (--CH_3) losses yielded the fragment ions at m/z 293 and C–C bonds cleavage gave m/z 103 and m/z 205 as a characteristic ions fragments while the ion at m/z 117 is originate from a TMS residue. The 100 urine samples from athletes and non athlete volunteers were tested and showed that 80% of the participants have a urinary glycerol concentration ≤ 20µg/ml as demonstrated in (figure 2a). These results showed no difference comparing the result obtained from previous study done in Germany to assess the urinary glycerol levels which founded that 85% of the whole tested samples were below the 20µg/ml [12]. This 20µg/ml came from the ordinary lipolysis process in the human body [1]. As demonstrated in (Figure 2b) the majority of glycerol concentration of athletes samples (68%) accumulate in 5-20 µg/ml range, where non athlete volunteers have (54%) of the total tested samples in 5-20 µg/ml range. However (42%) of total

samples in the non athlete volunteers gave glycerol concentration less than 5.0 µg/ml. Sport performance and increased lipolysis during exercise may be the cause that influenced the high urinary glycerol concentration in athletes compared to non athlete volunteers. This can explain appearance of 3 samples with a urinary glycerol concentration between 40 -60 µg/ml in the athletes group (Figure 2a). Result showed slightly higher concentration from athletes who gave urine samples after physical exercise; depend on endogenous glycerol which is resulted from increase lipolysis and fat metabolism that yielded higher urinary glycerol concentration compared with samples from athlete volunteers at rest [(1)]. The evaluation of glycerol level regard to sport kind showed that 34% of the athlete volunteers with a urinary glycerol concentration in 10 – 20 µg/ml range were an endurance sport performers. Where is a strength sport performer's yielded lower glycerol concentrations with a range 5 – 10µg/ml. Game sport which has the largest samples percentage by 46% of all tested samples exhibited relatively higher urinary glycerol levels comparing with the other sport (Figure 2b). Hubbly bubbly effect on the urinary glycerol level for the 20 participants showed insignificant changes in the baseline of urinary glycerol levels as in (Figure 2c). So, hubbly bubbly smoking can`t contributed in urinary glycerol concentration and as consequence can`t be used for glycerol doping which is prohibited by the WADA.

Conclusion

Urinary glycerol levels of 100 samples divided between athletes and non athlete volunteers clarified that the normal urinary glycerol concentration not exceeded the 40 µg/ml for the non athlete population and hardly reached 60 µg/ml urinary glycerol concentrations in the athlete group. In general, 80% of the two target population (athletes and non athlete) volunteers showed normal urinary glycerol concentration ≤ 20µg/ml. The variation in the endogenous glycerol concentration can influence by the volunteers physiological fat storage and metabolism, amount or type of exercise, physical performance and food intake quality. Based on the study outcome, the threshold for urinary glycerol to detect glycerol doping must be set lower than 1mg/ml that determined by the WADA; in order to detect glycerol doping practice more efficiently and insure prevention any case of glycerol doping practice

On the other hand hubbly bubbly smoking regime used in Jordan cannot contribute in increasing urinary glycerol concentration. Therefore no hyperhydration effect can happened after hubbly bubbly smoking. However, limitation such as control diet of the participants and smoking before conducting the study must be done, but due to time and the participants' commitment issues it was not possible to fill this gap during this study.

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Authors Column

Shrouq Ismail Ali Mahameed did her Masters in Analytical Toxicology from Faculty of Medicine of the University of Jordan, Amman /Jordan.

She was Pharmacist in Hebron, Palestine (2010 – 2011), Teacher assistant , Toxicology Division, Faculty of Medicine of the Jordanian University (2012-2013) and thereafter Trainee , Toxicology laboratory of the Jordanian University Hospital.