Vol. 7, No. 4, 2016
ISSN 2233-4203/ e-ISSN 2093-8950
LETTER www.msletters.org | Mass Spectrometry Letters

# Analysis of Glycerol with Isolation of Endogenous Interferences using "Dilute and Shoot" Strategy and High-Resolution Mass Spectrometry in Human Urine for Antidoping Testing

**Yongseok Kim,[1,2] Hophil Min,[1] Changmin Sung,[1] Ju-hyung Park,[1] Junghyun Son,[1] Kang Mi Lee,[1] Ho Jun Kim,[1] Jaeick Lee,[1] Oh-Seung Kwon,[1] and Ki Hun Kim[1]***

[1]*Doping Control Center, Korea Institute of Science and Technology, Hwarang-ro 14-gil 5, Seongbuk-gu, Seoul 02792, Republic of Korea*
[2]*Department of Chemistry, Yonsei University, Yonsei-ro 50, Seodaemun-gu, Seoul 03722, Republic of Korea*

*Received November 30, 2016; Revised December 14, 2016; Accepted December 19, 2016*
*First published on the web December 31, 2016; DOI: 10.5478/MSL.2016.7.4.000*

**Abstract :** Glycerol was identified and isolated from endogenous interferences during analysis of human urine using high-resolution mass spectrometry (HRMS) for doping control. Urinary sample preparation was simple; the samples were diluted with an organic solvent and then analyzed using a liquid chromatography-mass spectrometry ("dilute and shoot" method). Although the interfering ion peaks were observed at the similar retention time of glycerol, the inference could be identified by isolation with HRMS and further investigation. Thus, creatinine was identified as the endogenous interference for glycerol analysis and it also caused ion suppression resulting in the decrease of glycerol signal. This study reports the first identification and efficient isolation of endogenous interferences in human urine for "dilute and shoot" method. The information about ion suppression could be novel to prevent overestimation or a false result for antidoping analysis.

**Keywords :** Mass spectrometry, Doping control, Dilute and shoot, Orbitrap, Glycerol analysis

## 1. Introduction

The World Anti-Doping Agency (WADA) regulates the list of prohibited substances and requires that an accredited lab should analyze substances with appropriate methods. Various prohibited substances were classified to 11 classes including anabolic steroids, beta-2 agoinsts, diuretics, stimulants, masking agent, etc.[1] Glycerol known to regulate osmosis in human body can induce increment of plasma by osmosis.[2] Therefore, it can lower count of erythrocyte or hemoglobin concentration, which provides a role of 'masking agent' to interfere detection of drugs for hematogenesis such as erythropoietin. The oral dosing or intravenous injection of glycerol was prohibited by WADA from 2010 and 4.3 mg/mL of urinary concentration of glycerol is its threshold for a positive result.

The analysis of glycerol has been performed by colorimetric test or chromatography-mass spectrometry. The colorimetric test is based on measuring a formaldehyde concentration after reaction of glycerol oxidation with periodate anion.[3] Although this method has cost benefit, some issues remain. It takes a large volume of reagents and sample, and only sugar alcohols can be analyzed by this test. Otherwise gas chromatography-mass spectrometry (GC-MS) method could provide high sensitivity and accuracy but it requires a long reaction time for derivatization.[4] Currently, a method using liquid chromatography-mass spectrometry (LC-MS) has been developed. Dong et al.[5,6] reported a method using derivatization by benzoyl chloride in *n*-hexane and it showed high selectivity by product ion scanning. Since this method is accompanied by an additional reaction and extraction steps, a simple method is required that can be included in a routine screening method for multi-target analysis covering almost all classes in a single LC-MS sequence for doping control.[7] Recently, a strategy known as the "dilute and shoot" method has been gained much attention as a screening test procedure, which involves direct injection of target analytes in a diluted urine without any extraction or concentration step. This method can be applied successfully in polar compounds or metabolites that are difficult to be extracted or detected.[8] This strategy was

*Reprint requests to Ki Hun Kim
E-mail: kihun.kim@kist.re.kr

successfully demonstrated for selected compounds in diuretics, stimulants, narcotics, plasma volume expander, and beta-2-agonists.[9-12] Although "dilute and shoot" method can provide various advantages, some problems persist concerning an ionization suppression[13] or a spectral congestion.[14] in a mass spectrometric analysis.

In this study, we developed a method for glycerol screening based on "dilute-and-shoot" and identified an ion suppression effect and an interference by ions having similar m/z value and retention time. The main component of interference was firstly identified to creatinine by further investigation and database searching, and its ion suppression was discussed according to creatinine concentration. The creatinine isotope of similar m/z value to glycerol was successfully separated by high-resolution mass spectrometry (HRMS) and the ion suppression effect was observed in various concentrations of creatinine. Method validation was also performed for routine screening method for antidoping analysis.

## Experimental

### Chemicals and reagents

Glycerol, $d_5$-glycerol, sodium bicarbonate and creatinine were obtained from Sigma (St. Louis, USA) Methanol was purchased from J.T. baker (Center Valley, USA), and formic acid was obtained from Kanto chemical (Tokyo, Japan). All reagents were of analytical grade. The water was purified using Aqua MAX from Millipore (Darmstadt, Germany) for the mobile phase of chromatography and sample preparation.

### LC–MS/MS analysis

The target substances were separated via a UFLC XR series high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) using a Kinetex C18 column (100 mm × 2.1 mm I.D., 2.6 μm particle size; Phenomenex, Torrance, USA) connected to a guard column (2.1 mm I.D.). The mobile phase comprised 0.1% aqueous formic acid solution for mobile phase A and 0.1% formic acid in methanol for B. Gradient elution was applied at a flow rate of 0.5 mL/min, and the initial condition of 2% mobile phase B was held for 0.5 min, ramped to 95% B over 8.5 min, and then maintained for 9.0 min. Subsequently, re-equilibration for 1 min at 2% B was performed, thereby giving an overall runtime of 10 min. The mass spectra were obtained via a TSQ Quantum triple quadrupole mass spectrometer and Q Exactive plus tandem mass spectrometer from Thermo Scientific (San Jose, USA) in a positive-ion mode, and the capillary temperature was set to 300 $^{o}$C. The flow rates of the sheath gas, ion sweep gas, and aux gas were 53 arb (arbitrary unit), 3 arb, and 14 arb, respectively. The ion-spray voltage was 4000 V and the mass spectra acquisition was performed selective ion monitoring (SIM) for glycerol and its isotope. The resolution of a mass spectrometer highly affects the scan rate in terms of the time-domain transient in Fourier-transform MS such as Orbitrap. Therefore, the initial resolution was set to the lowest value (FWHM 17,500) to obtain a fast scan rate.

### Sample preparation

To prepare a reference sample, a stock solution of glycerol (43 mg/mL) was diluted in 90 μL of mobile phase A and then 10 μL of solution including $d_5$-glycerol (43 mg/mL) and methaqualone (1 μg/mL) in methanol was spiked as an internal standard (ISTD) to the threshold concentration established by WADA, which was 4.3 mg/mL. The urine sample (positive control urine, PCU) was prepared via removal of particles from the pooled urine (negative control urine). Particles were removed via centrifugation of 300 μL of urine at 10,000 g for 10 min, and then 80 μL of supernatant was transferred to an autosampler vial. Subsequently, 10 μL of glycerol solution and 10 μL of ISTD solution were spiked. Each sample was vortexed, and then 5 μL of sample was injected into an HPLC column. No significant difference was observed in retention time or separation efficiency by presence of methanol in a sample.

## Results and Discussion

### Isolation of glycerol ions from urine sample

This study was a first successful identification and isolation of the endogenous interference from target compounds using "dilute and shoot" method and LC-HRMS. The sodium adduct of glycerol ($[M+Na]^+$) was only detected and it did not produce product ions through collision-induced dissociation (CID); therefore, glycerol analysis was performed via MS. Glycerol showed a relatively low signal intensity in the urine sample than in the reference sample, and this result was considered as the high matrix effect from urine. Among them, it was identified that creatinine exerted large influence on the glycerol screening result obtained via MS and presence of creatinine was confirmed by an additional analysis (data not shown) and database searching via mzCloud from Thermo (www.mzcloud.org). The extracted chromatograms of glycerol and creatinine were shown in Figure 1a and they were detected at the similar retention time (0.48 min for glycerol and 0.58 min for creatinine). Creatinine was detected with very high intensity in the mass spectrum at 0.48 min., whereas isotope of creatinine and glycerol were observed with relative low intensity as shown in figure 1b. The abundance of creatinine isotope (m/z 115.0702) was greatly lower (< 5%) than monoisotope of creatinine but it was detected with higher intensity than glycerol. The difference of m/z value between glycerol and creatinine isotope was only 0.0328 and 4,000 or more resolution is required to resolve two peaks. Therefore, glycerol peak

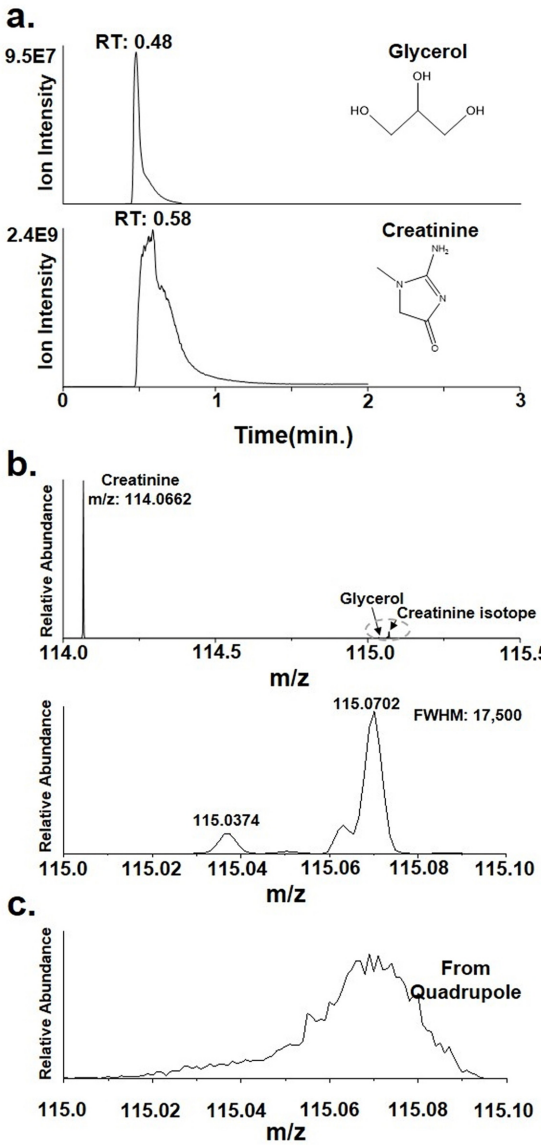


Figure 1. (a) The extracted chromatograms of glycerol and creatinine. Glycerol was detected as a sodium adduct ($[M+Na]^+$) of m/z 115.0374 and creatinine was detected as a protonated ion of m/z 114.0662. (b, top) The mass spectrum at the retention time of 0.48 min. The most abundant ion was creatinine but glycerol and creatinine isotope were observed with trace intensity. (bottom) the enlarged spectrum for glycerol and creatinine isotope (the dashed circle in the upper spectrum). Glycerol and creatinine isotope ions showed similar m/z value (115.0374 and 115.0702) but they were successfully separated with FWMH 17,500 by HRMS. (c) The mass spectrum of glycerol and creatinine mixture from a quadrupole mass spectrometer (Thermo TSQ) for comparison of separation efficiency in mass spectrometry. The sample and chromatography condition was equal to prior result (figure a and b). The separation of glycerol and creatinine ions was not sufficient by a resolving power of the triple quadrupole mass spectrometer, therefore no significant peak of glycerol was observed.

was thoroughly isolated in the Orbitrap mass spectrometer with FWHM 17,500 as shown in the enlarged spectrum of Figure 1b. Thus, it was identified that the interfering compound in glycerol analysis was the isotope of creatinine, and its influence was successfully removed via HRMS for an accurate screening. For comparison, the mass spectrum of same sample from a triple quadrupole mass spectrometer (TSQ Quantum from Thermo) was described in Figure 1c, and the peak of glycerol was not observed in the spectrum due to insufficient isolation from a creatinine isotope. Therefore, it is considered that a high-resolution mass spectrometer such as an Orbitrap is required to analyze glycerol in a human urine.

**Ion suppression by high concentration of creatinine**

The ion suppression effect was observed in the extracted chromatogram of glycerol in a urine sample. Ion suppression of electrospray ionization could be occurred by high concentration of nonvolatile solute including analytes, especially in a complex matrix such as plasma or urine. Firstly, the creatinine concentration in urine sample was measured by a standard addition method, and it was 1.1 mg/mL (data not shown). It was corresponded to a normal level of healthy human (0.5 ~ 2.0 mg/mL). To identify the effect of creatinine in ion suppression, reference samples were prepared by spiking various concentration (0~5.0 mg/mL) of creatinine at a fixed concentration of glycerol (4.3 mg/mL) in water, then analyzed. Some of chromatograms were described in Figure 2a. The peak area of glycerol was decreased as creatinine concentration was increasing, and the time range of glycerol signal decreasing corresponded to the retention time of creatinine. The plot of correlation between a glycerol peak area and creatinine concentration was described in Figure 2b. The glycerol peak area was rapidly decreased in a range of 0~0.5 mg/mL of creatinine concentration but there were no significant changes above 4.0 mg/mL of creatinine, therefore the ionization was considered to be saturated by creatinine at that point. With this result, it is considered that ion suppression was affected by presence of high amount of creatinine. Although ion suppression effect was present in glycerol analysis, but the screening was thoroughly available for a threshold concentration of glycerol by this method.

**Method validation**

The method was validated for qualitative purpose or screening considering the parameters including selectivity, matrix effect, intra- and inter-day precision and limit of detection (LOD) as required from ISO/IEC 17025 and WADA guidelines. Selectivity was validated by analysis of spiked five urine samples and there was no significant interfering peaks or retention time differences. Matrix effect was evaluated by analyzing five replicates of positive control urine (PCU, 4.3 mg/mL of glycerol)

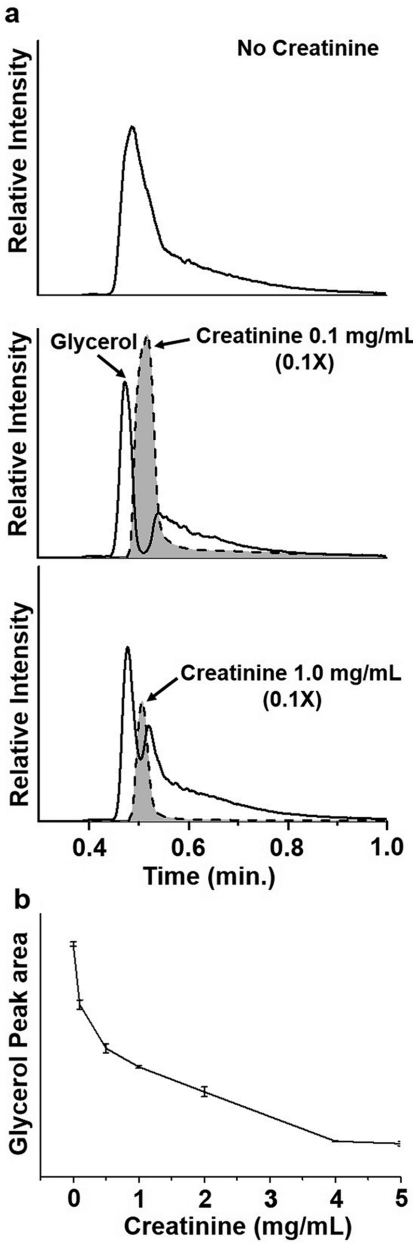


Figure 2. (a) The chromatograms of reference samples with a fixed concentration of glycerol (solid line) and various concentration of creatinine (1.0, 0.1 and 0 mg/mL, dashed line with filled) for observation of ion suppression by creatinine. The signal of glycerol was decreased at same time range to the retention time of creatinine and the suppression effect was according to creatinine concentration. The peak of creatinine was reduced to 1/10 for easier comparison to glycerol in a single chromatogram. (b) The plot of glycerol peak area according to a creatinine concentration in the samples ($n = 5$ for each concentration). The trend was showing glycerol peak area was decreasing dramatically when creatinine was spiked at a concentration of 0 to 0.5 mg/mL, but there was no large difference in the peak area when more than 4.0 mg/mL of creatinine was spiked.

sample and glycerol standards of equal concentration diluted in water containing sodium. With this result, matrix effect was calculated (peak area from PCU / standard in water) and it was 29.4%. Validation of precision (intra- and inter-day) was determined by analyzing 6 replicates of three different concentrations in a single day and three days ($n = 6/6/6$ and 18/18/18), and then coefficient of variation (CV) of peak area was calculated and all values were less than 25%. For determination of LOD, five PCU samples were prepared at each concentration (from 0.1 μg/mL to 4.3 mg/mL) and analyzed. LOD was determined by the lowest concentration with a signal to noise ratio of 3:1 and showing 25% of CV or less and it was 10 mg/mL.

## Conclusions

In this study, we observed some spectral congestion caused by endogenous interferences from human urine in the analysis of glycerol using "dilute and shoot" strategy, and the glycerol peak was successfully isolated via an HRMS. Furthermore, interfering compound was identified as an isotope of creatinine via database searching and further investigation. Creatinine also could be a cause of ion suppression that was identified by a correlation between the glycerol peak area and the creatinine concentration. Although the ion suppression was present, the analysis was available and the method was validated for a routine screening experiment. We anticipated that the results can provide novel information to prevent underestimation or false result for an antidoping analysis. Further expansion through the addition of various compounds is needed for a comprehensive screening assay for doping control.

## Acknowledgment

This work was supported by an intramural grant from Korea Institute of Science and Technology.

## References

1. WADA. *The 2016 prohibited list international standard*, https://www.wada-ama.org/en/international-standards. **2016**.
2. Polyviou, T. P.; Easton, C.; Beis, L.; Malkova, D.; Takas, P.; Hambly, C.; Speakman, J. R.; Koehler, K.; Pitsiladis, Y. P. *Nutr.* **2012**, 4, 1171.
3. Nash, T. *Biochem. J.* **1953**, 55, 416.
4. Thevis, M.; Guddat, S.; Flenker, U.; Schänzer, W. *Eur. J. Mass Spectrom.* **2008**, 14, 117.
5. Dong, Y.; Yan, K.; Ma, Y.; Yang, Z.; Zhao, J.; Ding, J. *J. Chromatogr. B* **2016**, 1022, 153.
6. Dong, Y.; Ma, Y.; Yan, K.; Shen, L.; Wang, X.; Xu, Y.; He, G.; Wu, Y.; Lu, J.; Yang, Z.; Feng, F. *J. Chromatogr. B* **2014**, 957, 30.

7. Jeong, E. S.; Kim, S. H.; Cha, E. J.; Lee, K. M.; Kim, H. J.; Lee, S. W.; Kwon, O. S.; Lee, J. *Rapid Commun. Mass Spectrom.* **2015**, 29, 367.
8. Görgens, C.; Guddat, S.; Orlovius, A. K.; Sigmund, G.; Thomas, A.; Thevis, M.; Schänzer, W. *Anal. Bioanal. Chem.* **2015**, 407, 5365.
9. Jiménez Girón, A.; Deventer, K.; Roels, K.; Van Eenoo, P. *Anal. Chim. Acta* **2012**, 721, 137.
10. Guddat, S.; Solymos, E.; Orlovius, A.; Thomas, A.; Sigmund, G.; Geyer, H.; Thevis, M.; Schänzer, W. *Drug Test. Anal.* **2011**, 3, 836.
11. Fitzgerald, R. L.; Griffin, T. L.; Yun, Y. M.; Godfrey, R. A.; West, R.; Pesce, A. J.; Herold, D. A. *J. Anal. Toxicol.* **2012**, 36, 106.
12. Beck, O.; Ericsson, M. *Bioanalysis* **2014**, *6*, 2229.
13. Annesley, T. M. *Clin. Chem.* **2003**, 49, 1041.
14. Schlittenbauer, L.; Seiwert, B.; Reemtsma, T. *Rapid Commun. Mass Spectrom.* **2016**, 1560.