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Original Article

Grayanotoxin levels in blood, urine and honey and their association with clinical status in patients with mad honey intoxication

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ABSTRACT

Objectives: The purpose of this study was to investigate whether there is an association between grayanotoxin levels in urine and blood of patients with mad honey intoxication and in the honey consumed, and the resulting clinical picture. The pilot data acquired from this study was analysed in National Forensic Service, Daejeon Institute, South Korea and first results were published as a preliminary study.

Patients and methods: This descriptive study was conducted at a university hospital emergency department in Turkey. 25 cases diagnosed with mad honey intoxication were obtained the study. Samples of mad honey consumed by patients were obtained. Blood and urine specimens were collected at presentation to the emergency department. GTX 1 and GTX 3 levels from patients' blood, urine and honey consumed were investigated simultaneously using the LC-MS/MS system.

Results: Mean GTX 1 concentration in blood was 4.82 ng/mL and mean GTX 3 level 6.56 ng/mL. Mean GTX concentration in urine was 0.036 µg/mL and mean GTX 3 level 0.391 µg/mL. Mean GTX I concentration in honeys consumed was 8.73 µg/gr and mean GTX 3 level 27.60 µg/gr.

Conclusion: This descriptive study is show grayanotoxin levels in body fluids of patients with mad honey intoxication. No association was determined between grayanotoxin levels in blood and clinical data.

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1. Introduction

Mad honey, of which Turkey is the largest producer worldwide, is a natural product resulting from collection by honeybees of nectars and pollens from *Rhododendron ponticum* and *Rhododendron luteum* from the family Ericaceae.¹ No previous studies have reported the levels of GTX in the body fluids of individuals with mad honey intoxication. Due to its toxic effects, studies to date have concentrated on determining and characterizing GTX levels from plant and honey specimens. Liquid chromatography-mass

spectrometry (LC-MS/MS) in the last 20 years and liquid chromatography time-of-flight mass spectrometry LC-MS/MS more recently have made it possible to determine GTX from biological materials (leaves, flowers and honey).² Holstege et al. developed LC-MS/MS a rapid method of quantitative determination of GTX-I, GTX-II and GTX-III in biological specimens, including intestinal contents, stool and urine. Compound identification with this method is based on positive ion electrospray ionization and ion trap mass spectrometry. This sensitive technique is also capable of use for toxicology and associated laboratory investigation.^{3,4}

The purpose of this study was to determine GTX levels in urine and blood of patients with mad honey intoxication and in the honey consumed, and to investigate whether these are associated with clinical status. The pilot data acquired from this study was analysed in National Forensic Service, Daejeon Institute, South Korea and first results were published as a preliminary study.⁵

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2. Material and methods

This descriptive study was performed in a university hospital emergency medicine department. Approval was granted by the local clinical research ethical committee (permission no: 24237859-293). Chemical analysis of specimens was performed in the National Forensic Service, Daejeon Institute, South Korea.

2.1. Collection of mad honey, blood and urine specimens

Twenty-five cases applying to our university hospital emergency medicine clinic and diagnosed with mad honey intoxication were included in the study. Patients' histories and drug use histories were investigated. Patients with a history of heart disease and antiarrhythmic, antihypertensive drug use were planned to be excluded from the study. Specimens of mad honey consumed by patients diagnosed with GTX intoxication at the emergency medicine clinical between 2013 and 2014 were obtained. Patients' blood pressure and heart rate values were recorded for the comparison of GTX levels and clinical data. Systolic blood pressure and heart rate cut-off values were used for this comparison, in line with Advanced Cardiac Life Support (ACLS) guideline treatment recommendations.

Clinical data were recorded simultaneously at time of presentation. Blood (5 mL) and urine (5 mL) specimens were collected on presentation to the emergency department. GTX levels from patients' blood and urine levels and from honey consumed were investigated simultaneously. Blood and urine specimens were centrifuged at 5 °C at 12,000 rpm. Supernatants were collected after centrifuging and lyophilized overnight at –50 °C. Dry materials obtained were then stored at –80 °C until analysis.

2.2. GTX standard and specimen preparation

Cho et al.'s method, as confirmed by U.S. Food and Drug Agency (FDA) 2001 bioanalytical method validation guidance was employed. GTX standards and specimens were prepared in the same center as that used in Cho et al.'s study, using the high specificity and sensitivity method described by them, with confirmed accuracy, with minor modifications.⁶

2.2.1. Preparation of GTX standard

Standard stock solution was obtained by dissolving 1 mg GTX-I and GTX-III in 10 mL methanol (v/v). Internal standard (IS) stock solution was obtained by dissolving 1 mg clindamycin in 10 mL deionized water. GTX study solutions were obtained by dissolving stock solutions with the requisite concentration of methanol. IS was prepared by dilution to a concentration of 25 ng. Calibration standards were prepared by adding 0.5 mL GTX-I and GTX-III solutions to blood, urine and mad honey specimens (0.2 g). GTX concentrations in blood, urine and honey specimens were 10, 20, 50, 100 and 500 ng/mL. IS (25 ng/mL, 0.05 mL) was added at a fixed level to obtain a final concentration of 2.5 ng/mL. All solutions were stored at 4 °C until assay.

2.2.2. Preparation of blood specimens

0.05 mL IS (25 ng/mL), 0.5 mL phosphate buffer (0.05 M, pH 6) and 2 mL acetonitrile were added to 0.5 mL of all blood specimens in closed tubes. Specimen mixtures were centrifuged at 10,000 rpm, first for 3 min and then for 5 min in a vortex mixer. Supernatant solutions obtained were evaporated under N₂ at 60 °C. The residue remaining after evaporation was mixed with a vortex with the addition of 1 mL water. The resulting supernatants were transferred to adance:1 PEP cartridges previously washed with 1 mL water and 1 mL water. Following transferal, cartridges were washed with 1 mL water and 1 mL hexane. Cartridges were next

washed with 2 mL methanol and vacuumized. The liquid solution was then evaporated at 60 °C under N₂. Finally, 0.1 mL 1% acetic acid was added to the dry residue. After mixing with vortex for 1 min this was transferred to 5 µL aliquot tubes and loaded onto the LC-MS/MS system.

2.2.3. Preparation of urine specimens

0.05 mL IS (25 ng/mL) and 0.45 mL phosphate buffer (0.05 M, pH 6) were added to 0.5 mL of all blood specimens in tubes. Specimen compounds were mixed for 3 min in a vortex mixer. The resulting supernatants were transferred to SPE (adance:1 PEP) cartridges previously washed with 2 mL methanol and 2 mL water. Cartridges were washed with 2 mL water and finally with 60 °C under N₂. Finally, following evaporation, 0.1 mL 1% acetic acid was added to the dry remnant. After mixing for 1 min in a vortex, this was transferred to 5 µL aliquot tubes and loaded onto the LC-MS/MS system.

2.2.4. Preparation of honey specimens

Part of each honey specimen was placed into 10 mL mass flask tubes and carefully weighed to elicit 0.2 g. Next, 0.025 mL of IS was added to all specimens, and water was added to give a total volume of 10 mL. All specimens were mixed for 3 min in a vortex mixer. Next, 1 mL was taken from each honey specimen and placed into SPE (adance:1 PED) cartridges previously washed with 2 mL methanol and 2 mL water. Cartridges were then washed with 2 mL water and finally with 2 mL methanol. After vacuumization, the liquid solution obtained was evaporated at 60 °C under N₂. Finally, following evaporation, 0.1 mL 1% acetic acid was added to the dry remnant. After mixing for 1 min in a vortex, this was transferred to 5 µL aliquot tubes and loaded onto the LC-MS/MS system.

2.3. Liquid chromatography and mass spectrometer (MS) conditions

Throughout analysis, HPLC and LC-MS/MS working conditions published elsewhere by Cho et al. and validated in the latest publication were employed.⁶ GTX was analysed using an Agilent 1200 series (HPLC) (Agilent Technologies, Palo Alto, CA, USA) system. Chromatographic separation was performed at +40 °C with a Kinetex biphenyl column (2.6 µm, 100 × 2.1 mm i.d., Phenomenex, Torrance, CA, USA) protected by a C18 guard column (2.1 mm i.d., Phenomenex). Mobile phases consisted of 1% acetic acid in water (A) and 1% acetic acid in methanol (B). Gradient elution procedures were performed under injection with a flow rate of 0–13 min, 5–90% B; 13–20 min 90% B, 0.25 mL/min. The sampler was conserved at below 10 °C and used in a 5-µL injection volume.

Mass spectrometric identification was performed using a Sciex 3200 QTRAP (AB Sciex, Concord, Canada) system in positive ion mode. Analytes were analysed using multiple reaction monitoring (MRM) in positive ion transfer mode. Ion source temperature was maintained at 600 °C, and the spray voltage was adjusted to 5500 V. All source parameters were optimized under LC conditions, and electrical parameters were optimized with direct infusion. Analyst software (version 1.5.1, AB Sciex) was used for device adjustment, data collection and data analysis.

2.4. Calculation of GTX levels in blood, urine and honey specimens

Toxicokinetic parameters obtained using the LC-MS/MS system were calculated using WinNonlin software (version 5.2, Mountain View, CA, USA). Blood results were expressed as ng/mL, urine results as µg/mL and mad honey results as µg/g.

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