

# Examination using LC-MS/MS determination of grayanotoxin levels in blood, urine, and honey consumed by patients presenting to the emergency department with mad honey intoxication and relations with clinical data: a preliminary study

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**BACKGROUND AND OBJECTIVE:** Intoxications related to “mad honey” are frequently encountered in the Black Sea region of Turkey. Intoxication is established on the basis of whether honey was consumed when history was taken at presentation. The search for a simple and reliable method for showing the grayanotoxins (GTXs) in mad honey in body fluids and in honey consumed by patients is still at the research stage. The purpose of this preliminary study was to investigate GTX levels in blood, urine, and honey consumed by patients with mad honey intoxication and to determine whether there is an association with clinical status.

**DESIGN AND SETTINGS:** This descriptive study was conducted at the department of Emergency Medicine of Karadeniz Technical University Medical Faculty in Turkey. Mad honey, blood, and urine samples were obtained from patients between September 2013 and October 2014.

**METHODS:** Four cases presenting the Department of Emergency Medicine and diagnosed with mad honey intoxication were included in the study. GTX levels in blood, urine, and honey consumed by patients were determined using liquid chromatography–tandem mass spectrometry.

**RESULTS:** Patients’ mean blood GTX I level was 30.62 ng/mL, GTX III level 4.917 ng/mL, urine GTX I level 0.447 mg/mL, and GTX III level 1.998 mg/mL. The mean GTX I level in the honey samples consumed was 4.683 mg/g and GTX III level 8.423 mg/g.

**CONCLUSION:** The present study is unique in representing the first time that GTXs have been determined in human body fluids. There is now an urgent need for a large series of studies to provide statistical evidence whether there is a relationship between levels of toxins in human body fluids and clinical picture.

The term “mad honey” results from grayanotoxins (GTXs) in flowers of plants of the genus *Rhododendron L* (Ericaceae) being mixed in with honey by bees. Species containing the toxin include the purple-flowered *Rhododendron ponticum* and yellow-flowered *R. luteum*.<sup>1</sup>

Due to its toxic effects, researchers have to date concentrated on determining and characterizing GTX in

plant and honey samples, or on synthesizing or deriving novel GTXs. Holstege et al (2001) later developed a rapid liquid chromatography–tandem mass spectrometry (LC-MS/MS) method permitting liquid chromatography–tandem mass spectrometry the quantitative determination of GTX I, II, and III in biological samples including rumen contents, feces, and urine.<sup>2</sup>

The purpose of this study was to investigate GTX

levels in blood and urine and in honey consumed by patients with mad honey intoxication and to determine whether there is an association with clinical status. We also hope that clarifying the association between amounts of GTXs in the honey consumed and the patient's clinical status will help establish whether the honey that has been consumed is more or less toxic.

## METHODS

### *Collection of samples*

Four cases presenting to Emergency Medicine Clinic and diagnosed with mad honey intoxication were included in the study. Mad honey, blood, and urine samples were obtained from patients intoxicated with GTXs and presenting to the Department of Emergency Medicine at Medical Faculty. GTX levels in patients' blood, urine, and the honey they consumed were examined simultaneously. The blood (5 mL) and urine (5 mL) samples were obtained from patients on presentation to the emergency department. Patients' blood and urine specimens were centrifuged at 12000 rpm at 5°C. After centrifugation, the supernatants were collected and lyophilized at -50°C overnight, and the dry materials were stored at -80°C for further analysis.

### *Chemicals and reagents*

All chemicals and reagents were of analytical grade. GTX I was obtained from Professor Na of Chungnam National University, Korea. GTX III Hemi (ethyl acetate) adduct (purity,  $\geq 90\%$ ) and clindamycin hydrochloride (internal standard, IS) were purchased from Sigma-Aldrich (St Louis, Missouri). The solid-phase extraction (SPE) cartridge (based on the adsorption effect in the reverse mode) and adande:1 PEP (polymer enhanced polymer, 30 mg/mL), were purchased from Shiseido (Tokyo, Japan). HPLC-grade methanol and water were purchased from Fischer Scientific Co. (Fair Lawn, NJ, USA). Analytical reagent-grade glacial acetic acid was purchased from Merck Company (Darmstadt, Germany).

### *Preparation of GTX standards*

The validated method by Cho et al (2014) was followed for preparing GTX standard stock solutions. Standard stock solutions were prepared by dissolving 1 mg of GTX I and 1 mg of GTX III in 10 mL methanol (v/v), respectively. The stock solution for IS was made by dissolving 1 mg of clindamycin in 10 mL deionized water. Working solutions of GTXs were prepared by serially diluting stock solutions with methanol to required concentrations. IS were prepared by dilution with water to a

concentration of 25 ng/mL. Calibration standards were prepared by spiking each 0.05 mL working solution of GTX I and GTX III into 0.5 mL of the blood, urine, or mad honey samples (0.2 g). A fixed amount of IS (25 ng/mL, 0.05 mL) was added to each sample to achieve a final concentration of 2.5 ng/mL.

### *Sample preparation*

A 0.05-mL aliquot of IS (25 ng/mL), 0.5 mL of phosphate buffer, and 2 mL of acetonitrile were added to a 0.5-mL aliquot of blood sample in cap tubes. Sample blood mixtures were vortex-mixed for 3 minutes and then centrifuged at 10 000g for 5 minutes. The supernatant solution was transferred and evaporated under  $N_2$  at 60°C. The residue was then dissolved in 1 mL of water and vortex-mixed. All the supernatant was loaded onto a cartridge (adande:1 PEP) preconditioned with 1 mL of methanol and 1 mL of water. After complete loading, the cartridge was washed with 1 mL of hexane. GTXs were subsequently eluted with 2 mL of methanol. The eluate was evaporated under  $N_2$  at 60°C. The dried residue was reconstituted in 0.1 mL of 1% acetic acid in methanol.

A 0.2-g mad honey sample was precisely weighed into a 10-mL mass flask. Next, 0.025 mL of IS and water were added to make a total sample volume to 10 mL. Next, 0.05 mL of IS and 0.45 mL of phosphate buffer were added to a 0.5-mL urine sample. One milliliter of a well-mixed honey sample or urine sample was loaded onto an SPE cartridge preconditioned with 2 mL of methanol followed by 2 mL of water. After sample loading, the cartridge was eluted with 2 mL of methanol. The eluate was dried under  $N_2$  at 60°C, and the residue was reconstituted in 0.1 mL of 1% acetic acid in methanol.

Aliquots of 5  $\mu$ L of the clear supernatants were injected into the LC-MS/MS system.

### *Liquid chromatography and mass spectrometer (MS) conditions*

Throughout the analysis, optimized validated high-performance liquid chromatography (HPLC) and LC-MS/MS working conditions were selected based on a recent publication by Cho et al (2014).

Mass spectrometric detection was performed on a Sciex 3200 QTRAP system (AB Sciex, Concord, Canada) equipped with an electrospray ionization source in positive ion mode. Analyst (version 1.5.1, AB Sciex) software was used for instrument setting, data acquisitions, and processing.

## RESULTS

Three of the patients presented to the emergency department were male and 1 female. The majority of pa-

tients reported that symptoms began 2 hours after consuming honey for male patients and 30 minutes after consuming for female patients. All patients presented with dizziness and nausea; hypotension and bradycardia were present in all subjects in **Table 1**.

GTX I and GTX III levels in blood collected from patients presenting to our emergency department due to mad honey poisoning, and levels of these detected in the honey consumed are shown in **Table 2**. The highest blood GTX I and GTX III levels were determined in Case 1. The amount of GTX I in the honey consumed by this patient was 0.61  $\mu\text{g/g}$ . This patient's blood pressure and heart rate at presentation to the emergency department were 60/40 mm Hg and 42 beats/min, respectively.

Although our fourth case consumed only a small amount of honey, the symptoms and signs appeared more quickly, and the highest level of GTX III was also determined in the honey consumed by this patient.

The patients' mean blood GTX I level was 30.62 ng/mL, mean GTX III level was 4.917 ng/mL, mean urine GTX I was 0.447  $\mu\text{g/mL}$ , and mean GTX III level was 1.998  $\mu\text{g/mL}$ . The mean GTX I level in the honey consumed was 4.683  $\mu\text{g/g}$  and the mean GTX

III level was 8.423  $\mu\text{g/g}$ . The patients' mean blood pressure was 77.5/47.5 mm Hg and mean heart rate was 38 beats/min.

## DISCUSSION

The most common symptoms in Mad honey intoxication are pronounced hypotension and bradycardia.<sup>1</sup> Other common symptoms are sweating, stupor, altered mental state, syncope, diplopia, blurred vision, and hypersalivation.<sup>3</sup> The mean amount of honey consumed in mad honey intoxications has been reported at 5 to 30 g. Symptoms begin 1.5 to 3 hours after consumption.<sup>4</sup> Intoxicated patients generally exhibit improvement with support therapy, with saline solution and 1 to 2 mg of intravenous atropine. Patients are discharged 2 to 6 hours after cardiac monitoring in mild intoxications, while symptoms disappear after 24 hours at latest in more severe cases.<sup>1</sup>

It is generally very difficult to estimate statuses in patients presenting with "mad honey" intoxication. Which patients should be hospitalized and which monitored on an outpatient basis is, therefore, a significant problem that physicians are facing.<sup>5</sup> Knowing the amount of toxin in blood, urine, and honey consumed can be

**Table 1.** Patients' gender, time of onset of symptoms after honey consumption, amount of mad honey consumed, blood pressure, and heart rate. Data belong to patients presenting to the Department of Emergency Medicine at a university hospital (Turkey) with mad honey intoxication.

Case	Gender/Age (y)	Approximate time of onset of symptoms after consumption (h)	Amount of honey consumed <sup>a</sup> (g)/ source of honey <sup>b</sup>	Blood pressure (mm HG)	Heart rate (beats/min)
Case 1	Male, 67	2	108/Arsin	60/40	42
Case 2	Male, 51	2	150/Sürmene	90/60	40
Case 3	Male, 70	2	100/Of	80/50	35
Case 4	Female, 40	0.3	16/Yomra	80/40	35

<sup>a</sup>Amount of honey consumed by patients is not precise. Researchers weighed the amount of honey consumed as told by the patients.

<sup>b</sup>Townships around Trabzon city where the mad honey samples obtained intoxicated patients. Honey collected at altitudes of 1370 m (above sea level).

**Table 2.** Levels of grayanotoxins (GTX I and III) in mad honey, blood, and urine samples obtained from patients presenting to the Department of Emergency Medicine at a university hospital (Turkey) with mad honey intoxication.

Case	Honey (mg/g)		Blood (ng/mL)		Urine (mg/mL)	
	GTX I	GTX III	GTX I	GTX III	GTX I	GTX III
Case 1	0.61	2.525	86.20	8.187	0.162	1.904
Case 2	7.77	11.03	19.25	2.774	0.576	1.913
Case 3	0.451	2.977	8.130	7.014	0.186	0.796
Case 4	9.895	16.890	8.910	1.692	0.864	3.378
Mean	4.683	8.423	30.62	4.917	0.447	1.998

important to provide a relation between levels of toxins in human body fluids and clinical picture.

No previous studies have investigated blood and urine specimens and honey consumed by patients with such intoxications. The determination of GTXs was first achieved in some biological samples including feces, rumen, and urine (Holstege et al 2001). The difference of origin of the biological samples, and extraction and isolation of GTXs from these samples, owing to the lack of analytical sensitivity and low selectivity, required a complicated sample pretreatment procedure for extraction and purification.<sup>2</sup>

This uncertainty in the sampling, detection, and quantification of GTXs using blood samples was eliminated by Cho et al (2014), who for the first time developed a sensitive, reliable, and validated quantitative LC-MS/MS method in rat whole blood.<sup>6</sup> In the present study, methods described by Cho et al (2014) were applied to blood samples from the 4 cases presenting with mad honey intoxication to our emergency department. Samples of the mad honey responsible were also analyzed in parallel.

No significant correlations were determined between gender, time of onset of symptoms after honey consumption, amount of mad honey consumed, blood pressure or heart rate, and GTX I and GTX III contents, and several reasons may be attributed to this fact: (a) it may be due to age differences between the patients, the distances involved, geographical variation between the honey sources, and unequal gender distribution among the patients. (b) The distribution and abundance of the *Rhododendron* species, *R. ponticum*, and *R. luteum* are quite heterogenous at the altitudes (Table 1)

where the mad honey that caused intoxication was produced. This variation may affect GTX concentrations in nectar composition, and thus mad honey composition. (c) Variations in onset time and GTX concentrations mainly result from the speed of absorption as well as blood concentrations of GTX. (d) Large quantities of honey may disturb the absorption of GTX. Onset time may, therefore, be delayed. Another possible reason is that "mad honey" contains above 20 analogues. We along with other study groups detected only 2 or 3 kinds of GTXs. These difficulties can be resolved by providing GTX standards from the analogues as working standards.

In conclusion, this is a preliminary study, and the first to show GTX levels in specimens from human body fluids. No statistical comparison could be performed, as the case numbers were limited. There is now an urgent need for large series studies to provide statistical evidence whether there is a relation between levels of toxins in human body fluids and clinical picture. The research value of the present study lies in determining and quantifying GTXs obtained from human body samples, blood, and urine, and would assist researchers interested in GTXs in terms of forensics and clinical toxicology.

#### Conflicts of interest

*There are no conflicts of interest.*

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