

# Confirmation of Gelsemium Poisoning by Targeted Analysis of Toxic Gelsemium Alkaloids in Urine

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## Abstract

The gelsemium plants are highly poisonous but toxicological evaluation of suspected poisoning cases has been hampered by the chemical complexity of the gelsemium toxins involved. A novel liquid chromatography–tandem mass spectrometry protocol was optimized for the collective detection of gelsemine and related alkaloids from *Gelsemium elegans*. The screening protocol was applied to the clinical investigation of unexplained intoxications following the ingestion of seemingly nontoxic herbs. In three clusters of toxicological emergencies ranging from severe dizziness to respiratory failure, *Gelsemium elegans* mistaken for various look-alike therapeutic herbs was suspected to be the hidden cause of poisoning. Nine cases of gelsemium poisonings were thus ascertained by the diagnostic urine alkaloid profiles. Gelsemine was sustained as the main urinary marker of *Gelsemium* exposure.

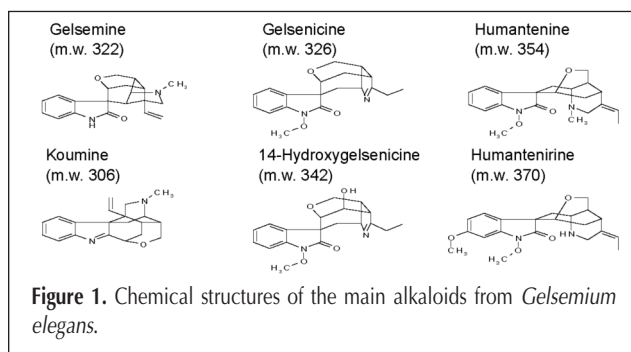
## Introduction

Gelsemium (Loganiaceae) is a small genus of three species, *G. elegans* Benth., *G. sempervirens* Ait., and *G. rankinii* Small. Of these, *G. elegans* is distributed in Southeast Asia and the two other species are native to North America. More than 50 indole/oxindole alkaloids with diverse chemical structures and biological activities have been isolated from these plants; the individual alkaloids present account for the variable toxicity observed between different local species, different parts of the plant, and different season of harvest (1,2). The alkaloids from *G. sempervirens* have been well characterized, with gelsemine (LD<sub>50</sub> ~56 mg/kg mice i.p.) being the most abundant and gelsemicine being the most toxic (LD<sub>50</sub> ~0.2 mg/kg rat i.p.) (3). A single case of pediatric poisoning by accidental ingestion of flowers from *G. sempervirens* has been reported (4).

*G. elegans*, which is known as “Gou-men” (lethal kiss) in China, has been used as traditional medicine for the treatment of pain, spasticity, and skin ulcers. However, the potent toxicity restricts its use to topical application only. In Hong Kong and other parts of China, the lethal plant has been notoriously used

in committing suicide and homicide (5,6). In addition, the gelsemium plant grows as a twining vine, and it may interweave with other edible plants, leading to inadvertent consumption. Typical symptoms of intoxication include rapid-onset dizziness, nausea, vomiting, blurred vision, limb paralysis, breathing difficulty, coma, and convulsion. In severe poisoning, life-threatening respiratory depression would lead to death (1). The alkaloids from *G. elegans* have also been studied extensively; the chemical diversity of the main gelsemium alkaloids is illustrated in Figure 1. The most abundant alkaloid is koumine (LD<sub>50</sub> ~100 mg/kg mice i.p.), which showed mild toxicity comparable to gelsemine. However, the latter isolated alkaloid gelsenicine (humantenine) proved to be the most toxic (LD<sub>50</sub> ~0.2 mg/kg mice i.p.) (7,8). Indeed, the fatal action of gelsenicine by respiratory inhibition in experimental animals (9) is analogous to that of gelsemicine (3), although the exact mechanism of their action appears controversial (1).

Despite numerous phytochemical studies on the gelsemium species, very limited information is available on the analysis of gelsemium toxins in biological samples. A high-performance liquid chromatography (HPLC) method for the separation of gelsemine, koumine, gelsenicine, 14-hydroxygelsenicine (humantenine), and humantenine has been described, but its application was restricted to the quality control of pharmaceutical extract from *G. elegans* (10). Gas chromatography–mass spectrometry (GC–MS) has been used to confirm gelsemine in the gastric content of a postmortem case of *G. elegans* poisoning (11), but the sensitivity would be insufficient for urinary toxin detection. Therefore, this study explores a liquid chromatography (LC)–tandem MS method targeting at the major alkaloids from *G. elegans*. This new method has



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been applied to studies of urine samples from clinical cases of suspected poisonings by the dangerous plant.

## Experimental

### Chemicals and reagents

Gelsemine was purchased from Sigma (St. Louis, MO). Stock solution of gelsemine was prepared by dissolving 10 mg of the compound in 10 mL 70% acetonitrile. Fresh plant of *G. elegans* (including leaves and stems) was collected from the Tai Po region (countryside of Hong Kong) in autumn and was authenticated by a qualified herbalist. The air-dried reference plant had been stored at ambient temperature for less than 18 months before analysis. The commercially available solvent extraction tubes, Toxi-Tube A, were obtained from Varian (Lake Forest, CA). The extraction tubes contained a solvent mixture of dichloromethane and dichloroethane and buffer salts of sodium carbonate and bicarbonate to give a pH of around 9 for urine samples.

### Patient subjects

Between March 2007 and February 2008, the laboratory received toxicological referrals of three unrelated clusters of

suspected gelsemium poisoning from local hospitals. The first episode involved a couple of spouse; the other two episodes involved two families of four members. All patients took self-prepared decoctions of different therapeutic herbs not known to cause acute toxicity; however, preliminary inspection of residual herbal materials was suggestive of gelsemium involvement (Table I). All patients recovered following medical treatment, but prolonged intensive care was required for Case 1, who exhibited life-threatening toxidrome (12). Urine samples were collected promptly from each patient; however, no urine sample was available from Case 2, who was discharged after a quick recovery. The residual raw plant related to the first episode, the dried plant material and residual decoction linked to the second episode, and the left-over decoction from the third episode were also submitted for analysis. As a standardized practice in the laboratory, raw plant materials from clinical cases were oven-dried for instant preservation.

### Extraction of plant materials, herbal decoctions, and urine samples

Dried plant samples were homogenized by sonicating 1 g grounded powder in 10 mL 70% acetonitrile at 37°C for 30 min. After centrifugation, 0.1 mL of the supernatant was diluted with 3.9 mL purified water prior to solvent extraction. Herbal decoction samples (0.5 mL) were diluted with 3.5 mL

**Table I. Clinical Summary of Suspected Gelsemium Poisoning Cases**

Case No.	Sex/ Age	Clinical Presentation	Intended Herbs	Source of Herb and the Part Consumed	Suspected Cause of Poisoning
1	F/65	Dizziness, generalized weakness, nausea, loss of consciousness, respiratory failure	<i>Mussaenda pubescens</i> (employed in the therapy of colds, tonsillitis, enteritis, and snakebites)	Raw leaves and stems freshly collected from countryside	Misidentification
2	M/69	Dizziness, generalized weakness, vomited out part of the herbs			
3	M/57	Dizziness, palpitation, blurred vision	<i>Tadehagi triquetrum</i> (used as an antipyretic, diuretic, and for promoting digestion)	Dried leaves and stems shared by relatives from rural area	Erroneous substitution
4	F/43				
5	M/17				
6	F/15				
7	M/50	Dizziness, palpitation, blurred vision, lower limb weakness	<i>Ficus simplicissima</i> (consumed as a traditional soup for dinner)	Dried root slices bought from nearby town	Contamination
8	F/43				
9	F/18				
10	F/13				

purified water prior to solvent extraction. Undiluted urine samples (4 mL), diluted decoction samples (4 mL), or diluted crude plant solutions (4 mL) were extracted by mechanical shaking in separate Toxi-Tubes for 20 min. After phase separation and solvent evaporation, the dried residue was reconstituted in 1.0 mL 50% acetonitrile and 0.2% formic acid in water for HPLC injection (20  $\mu$ L).

### LC-MS-MS protocol

An API 2000 LC-MS-MS triple-quadrupole MS (Applied Biosystems, Foster City, CA) with a TurboIonSpray ionization probe source was used. Purified nitrogen, which was obtained from a NM30L nitrogen generator (Peak Scientific, Billerica, MA), was used as the nebulizing gas and the collision gas. The HPLC platform series 200 (Perkin-Elmer, Waltham, MA) included two micropumps, an autosampler, a solvent mixer, and a degaser. Chromatographic separation was carried out at ambient temperature on a Zorbax Eclipse XDB C8 column (150 x 4.5-mm i.d., 5- $\mu$ m particle size, Agilent Technologies, Santa Clara, CA). Samples were eluted with gradient mobile phase by proportional mixing of two solvent delivered at a total flow rate of 0.5 mL/min: solvent A contained 0.2% formic acid in purified water, and solvent B contained 0.2% formic acid in acetonitrile. Solvent gradient conditions changed linearly from 10% B to 90% B in 10 min, stayed at 90% B for 3 min, and returned from 90% B to 10% B in 3 min. Total run cycle was 20 min. The entire flow was directed into the ionization source without splitting.

Direct infusion of the standard gelsemine solution (diluted to around 100 ng/mL with 50% acetonitrile and 0.2% formic acid in water) was performed using the built-in syringe pump to optimize various MS parameters. The electrospray source was operated at positive ionization mode with electrospray voltage of 5000 V, source heater probe of 450°C. Optimized compound-specific parameters included declustering potential of 26 V, focusing potential of 350 V, entrance potential of -12 V, collision gas setting of 4 (arbitrary units), cell exit potential of 2 V. Settings of nebulizer gas, heater gas, and curtain gas were 40, 50, and 25 (arbitrary units), respectively. The triple-quadrupole MS was operated at the multiple reaction monitoring (MRM) mode. Unit resolution was used in both mass selection quadrupoles Q1 and Q3. Two looped experiments were thus optimized to generate larger mass fragments at a collision energy of 33 V and to generate smaller mass fragments at a collision energy of 49 V. The data cycle for 12 MRM pairs was 0.72 s with a dwell time of 50 ms for each mass transition. Total detection time was 15 min. Sensitive quantification of gelsemine was achieved by monitoring of the mass transition of  $m/z$  323 to  $m/z$  70, with a separate mass transition of  $m/z$  323 to  $m/z$  236 serving as the additional qualifier. In the absence of pure substances, the extract of gelsemium alkaloids from the reference *G. elegans* was used to establish mass fragmentation, HPLC separation and peak identification of main gelsemium alkaloids other than gelsemine. Thus, the tentatively identified alkaloids and the monitored transitions were koumine ( $m/z$  307 to  $m/z$  220 and  $m/z$  307 to  $m/z$  70), gelsenicine ( $m/z$  327 to  $m/z$  296 and  $m/z$  327 to  $m/z$  108), 14-hydroxygelsenicine ( $m/z$  343 to  $m/z$  312 and  $m/z$  343 to  $m/z$

108), humantenine ( $m/z$  355 to  $m/z$  309 and  $m/z$  355 to  $m/z$  122), and humantenirine ( $m/z$  371 to  $m/z$  340 and  $m/z$  371 to  $m/z$  164).

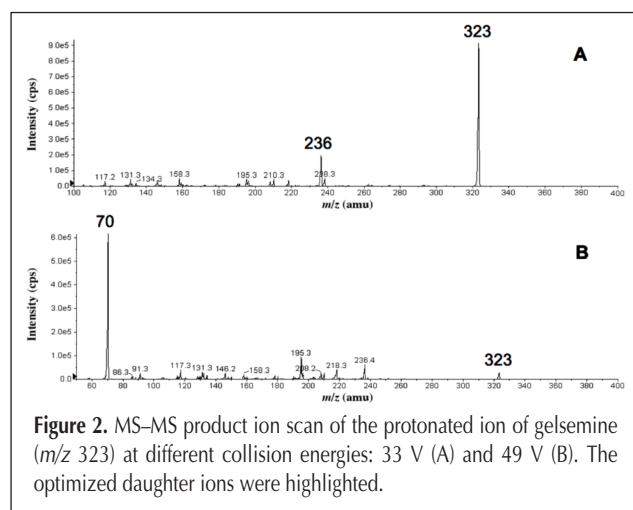
## Results and Discussion

### Electrospray mass spectra of gelsemine

Gelsemine was effectively detected by positive electrospray ionization (ESI)-MS. A simple solvent system with acetonitrile and formic acid was used throughout this study, which promoted the formation of the protonated ion of gelsemine  $[M+H]^+$  without common adduct ions from sodium or potassium. The fragmentation of the quasi-molecular ion of gelsemine is optimized at two different collision energies (Figure 2). Reliable identification of gelsemine was ensured by monitoring two specific mass transitions and its chromatographic retention time in the final LC-MS-MS protocol (13).

### Validation of gelsemine detection in urine samples by LC-MS-MS

Assessment of extraction efficiency was conducted by pre-extraction spiking of the standard (at 25 ng/mL) to random human urine samples. Ion-suppression effect was evaluated by post-extraction spiking of the standard (at 25 ng/mL) to different urine extracts. Mean extraction efficiency of gelsemine was 68 % (SD = 8%,  $n$  = 10). No significant ion suppression of gelsemine signal was observed in the post-extraction spiking experiment, with a mean analytical recovery of 97% (SD = 7%,  $n$  = 10). Detection limit of gelsemine was 0.5 ng/mL, with S/N > 5 (measured by peak height). Linear detector responses were recorded with five gelsemine levels from 1.0 to 150 ng/mL. For quantitative analyses of clinical samples based on external standardization, matrix-matched calibrator of gelsemine at 5.0, 50, and 125 ng/mL were prepared in pooled human urine to compensate for the anticipated extraction loss. Analyses of random urine samples spiked with gelsemine at 10 ng/mL showed an observed bias of + 9.5% and CV of 13% ( $n$  = 10), and those spiked with gelsemine at 100 ng/mL showed an observed bias of -0.5% and CV of 9% ( $n$  = 10).



**Figure 2.** MS-MS product ion scan of the protonated ion of gelsemine ( $m/z$  323) at different collision energies: 33 V (A) and 49 V (B). The optimized daughter ions were highlighted.

Testing of non-spiked random urine samples ( $n = 20$ ) confirmed absence of interfering signals.

### Study of main alkaloids in plant extracts of *Gelsemium elegans*

The optimized ESI-MS settings for gelsemine was applied as a crude screening of main gelsemium alkaloids in the extracts of the reference plant and the raw plant remnant from Case 1. Abundant ions detected by direct infusion of the plant extracts were consistent with the expected quasi-molecular ions of main gelsemium alkaloids characterized by previous phytochemical studies (1,2). A big difference between the relative abundance of these alkaloids in the two extracts was noticeable, which would be compatible with the wide divergence of alkaloid profiles reported (14–16). For instance, prominent ions in the extract of the reference gelsemium could possibly be attributed to koumine ( $[M+H]^+$  at  $m/z$  307), humantenine ( $[M+H]^+$  at  $m/z$  355), and humantenirine ( $[M+H]^+$  at  $m/z$  371); similar alkaloid profile has been reported in the gelsemium from Guangxi province of China (14). In contrast, dominant ions in the plant extract from case 1 could possibly be attributed to gelsenicine ( $[M+H]^+$  at  $m/z$  327) and 14-hydroxygelsenicine ( $[M+H]^+$  at  $m/z$  343), which were compatible with the principal alkaloids of gelsemium from Yunan province of China (16). To produce a gelsemium-specific LC–MS–MS fingerprint, the five target ions detected in the authentic gelsemium extracts were subjected to collision-induced fragmentations and chromatographic separation as standardized by gelsemine. As a result, an LC–MS–MS qualitative screening of main gelsemium alkaloids from *G. elegans* was defined in Table II.

Given the diversified cyclic skeletons attaching the indole/oxindole molecules and the lack of prior studies on the collision-induced fragmentation of these gelsemium alkaloids, a full structural correlation of the key mass fragments observed with the suspected alkaloids was not possible. However, based on the known electron-impact (EI) mass

fragmentation of these gelsemium alkaloids (1), a few common neutral losses and coincident mass fragments could be spotted (Table II). Proper interpretation of the ESI-MS–MS spectra of the complex molecules would demand advanced instrumentation capable of higher mass resolution and multi-stage MS. Unfortunately, gelsemine was the only gelsemium alkaloid of which a chemical standard was commercially available. Therefore, confirmatory and quantitative analyses of koumine, humantenine, humantenirine, gelsenicine, and 14-hydroxygelsenicine were beyond the scope of this study. Definitive identification of these gelsemium alkaloids would involve preparative purification of the alkaloids and structural elucidation by nuclear magnetic resonance spectroscopy (NMR) (17).

Nonetheless, the concept of multi-component fingerprinting of plant extracts by ESI-MS, MS–MS, or LC–MS–MS has been well-established in the quality control of medicinal products (18,19). It is believed that such fingerprinting techniques would also benefit the toxicological analysis of poisonous plant containing multiple toxins at variable quantities.

### Qualitative screening of major gelsemium alkaloids in urine

The LC–MS–MS screening procedure has been applied to all patient samples in this study; an example (case 3, Table I) is demonstrated in Figure 3.

The man took an herbal decoction intended for “dispelling

**Table II. LC–MS–MS Screening Parameters of Major Gelsemium Alkaloids**

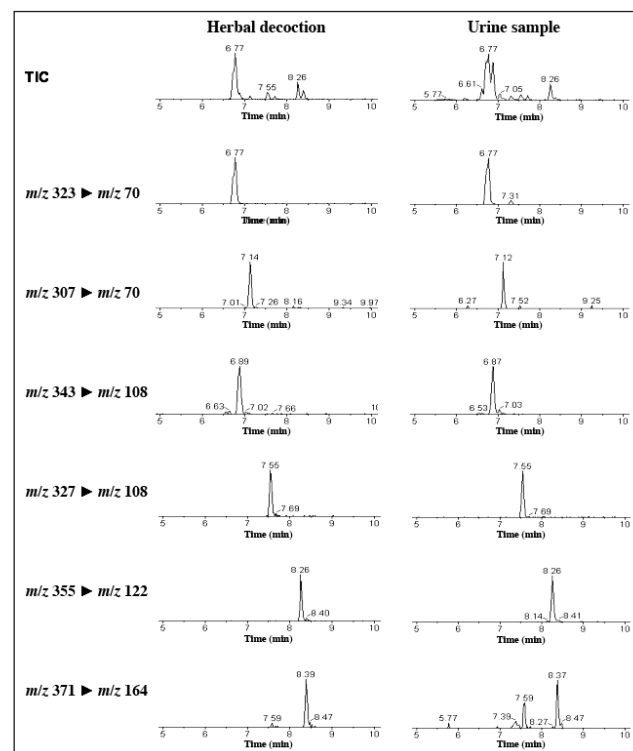
MS Mode	MS–MS Mode		Retention Time (min)	Gelsemium Alkaloids
	CE = 33 V	CE = 49 V		
Q1 ( $m/z$ )	Q3 ( $m/z$ )	Q3 ( $m/z$ )		
323	236	70	6.8	Gelsemine
307	220	70*	7.1	Koumine ? <sup>†</sup>
343	312 <sup>‡</sup>	108 <sup>§</sup>	6.9	14-Hydroxygelsenicine ?
327	296 <sup>‡</sup>	108 <sup>§</sup>	7.6	Gelsenicine ?
355	309	122*	8.3	Humantenine ?
371	340 <sup>†</sup>	164	8.4	Humantenirine ?

\* The same mass fragment was prominent in the electron-impact (EI) mass spectrum of the suspected alkaloid.

<sup>†</sup> ? = Tentative identification.

<sup>‡</sup> The mass fragment would be compatible with a neutral loss of the methoxy functional group (31 amu) attaching the indolic nitrogen of the suspected alkaloid.

<sup>§</sup> The common mass fragment of  $m/z$  108 from the parent ions of  $m/z$  327 and  $m/z$  343 was consistent with the two suspected alkaloids of related structures.



**Figure 3.** Application of the LC–MS–MS protocol to the toxicological investigation of suspected gelsemium poisoning (case 3 of Table I and Table III). Gelsemine and other tentatively identified alkaloids were detected in both the residual herbal decoction and the urine sample from the patient, confirming the diagnosis (the y-axis of the total ion chromatogram and the composite chromatograms by selected reaction monitoring were plotted as normalized signal abundance relative to the highest peak).



**Table III. Detection of Gelsemium Alkaloids in Clinical Cases**

Case No.	Sampling Time (hours post ingestion)	Urine Gelsemine Level (ng/mL)	Major Alkaloid Detected in Residual Herbal Samples
1	9	17	Gelsenicine (tentative identification)
3	3	128	Gelsemine
4	3	20	
5	3	54	
6	3	37	
7	2	21	Koumine (tentative identification)
8	2	28	
9	2	34	
10	2	10	

heat" but developed dizziness, palpitation, and blurred vision shortly thereafter. A urine sample was collected at the emergency room; all target gelsemium alkaloids screened positive. Importantly, gelsemine was abundantly excreted, consistent with its high content in the herbal decoction ingested. In addition, paired comparison of the alkaloid profiles from the urine and the residual herbal decoction showed significant changes in the relative abundance of target peaks. It would be logical to postulate that such changes may reflect the possible difference in human absorption, metabolism, or excretion of individual gelsemium alkaloids. However, evidence-based result interpretation is not possible, as no toxicokinetic data of any gelsemium alkaloid have been reported in the literature.

In effect, the LC–MS–MS fingerprinting technique, as standardized against gelsemine and validated by authentic gelsemium extracts, established a causal link between the ingestion of the toxic plant and the excretion of main alkaloids.

### Summary on the laboratory diagnosis of gelsemium poisoning cases

Results of the toxicological investigation of the three clusters of suspected gelsemium poisoning were summarized in Table III. Nine cases of gelsemium poisonings were thus established by the diagnostic LC–MS–MS alkaloid profile in urine. The laboratory confirmation is important not only for clinical diagnosis, but also for public health surveillance. As documented in this case series involving the misidentification, erroneous substitution, or contamination of various "cooling" herbs with *G. elegans* (Table I), the investigation of unexplained herbal intoxication presenting as acute neurological or respiratory depression should include the targeted screening for gelsemium alkaloids.

Gelsemine was detectable in all urine samples promptly collected from these acutely intoxicated subjects, despite the fact that it was not the major gelsemium alkaloids in all herbal samples involved. Urinary gelsemine excretion (Table III) did not correlate with clinical severity (Table I), as reflected in a six-fold difference in levels among the four family members of almost identical toxidrome (cases 3–6). Therefore, targeted

analyses of other gelsemium alkaloids with higher toxicity implicated in previous animal studies remain to be improved. Additionally, it would be worthwhile to explore whether blood concentrations of these gelsemium alkaloids would show a better correlation with the severity of poisoning (20).

## Conclusions

The present investigation is the first report on the detection of gelsemium alkaloids in urine specimens. Repeated observation on emergency poisonings confirmed that urinary gelsemine was a practical marker of gelsemium exposure in human subjects. The analytical protocols defined in this study, as well as the clinical diagnoses thus established, would be of reference value for future toxicological investigation.

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