Toxin Detection in Foods and Pharmaceutical Products

Food Toxin Programme: WHO case studies in mushroom poisoning

Recent development for authenticating Botulinum Neurotoxin in pharmaceutical products

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Government Laboratory
Hong Kong
Foodborne Toxin Analytical Service

Regular Services:
Mycotoxins
Histamine
Marine toxins
Mushroom toxins
Puffer fish toxins
Potato toxins
Natural toxins in herbs (Chinese medicine)

• To investigate & prevent food poisoning
• To protect public health for local community
Food Poisoning

Non-chemicals: bacteria, fungus, virus, parasites, etc.

Chemicals: toxins, pesticides, heavy metals, etc.
Foodborne Toxin Cases (2008 – 2013)

273 confirmed cases in Hong Kong, constituted ~8% of the total food poisoning cases
Calcium Oxalate Poisoning

Ca oxalate needles (raphides) found in edible vegetables, water spinach, water cress. Sharp needles cause pain, swelling in oral mucosa, burning sensation.
Mycetismus: WHO Cases

- Two reported fatal food poisoning cases in remote villages in the Indochina region
- Suspected eating wild poisonous mushrooms
- Local health authority claimed no capacity to test samples
- Western Pacific Regional Office contacted us for technical assistance
- Sending suspected remains of food for examination and analysis
Suspected Poisons (I)

Not intact, only 4 dark yellow fragments

Reported symptoms: dizziness; severe vomiting; diarrhea, abdominal pain & exhausted
Involved 13 victims, 4 died (5 to 10 days)
LC-MS/MS Analysis

An accredited method for detecting common toxins: amanitins, phallacidins and muscarine

Detection limit: 0.25 mg/kg
## Mushroom Toxin (mg/kg)

<table>
<thead>
<tr>
<th>Toxins</th>
<th>Parent Ion</th>
<th>MRM</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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</thead>
<tbody>
<tr>
<td>α-Amanitin</td>
<td>919.4</td>
<td>901.4*, 259</td>
<td>560</td>
<td>370</td>
<td>350</td>
<td>360</td>
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<tr>
<td>β-Amanitin</td>
<td>920.4</td>
<td>902.5*, 259</td>
<td>190</td>
<td>140</td>
<td>120</td>
<td>100</td>
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<tr>
<td>γ-Amanitin</td>
<td>903.4</td>
<td>885.3*, 243</td>
<td>0.9</td>
<td>1.6</td>
<td>0.9</td>
<td>0.8</td>
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<tr>
<td>Phallacidin</td>
<td>847.3</td>
<td>811.3*, 829</td>
<td>150</td>
<td>110</td>
<td>110</td>
<td>150</td>
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<tr>
<td>Phalloidin</td>
<td>789.3</td>
<td>753.3*, 771</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Muscarine</td>
<td>174.1</td>
<td>115.1*, 97</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Very high level of the most deadly α-amanitin was detected

Lethal dose: ~10 mg for average-sized adults

Presence in some toxic species of *Amanita* genus
Toxic Culprit

DNA analysis showed that it is *Amanita exitialis*

Known as Guangzhou destroying angel

- Onset of action: 6 – 18 hrs
- Latent period: 2 – 4 days (false recovery)
- Intoxication stage: liver and kidney failure, black urination, yellow skin, loss of strength
- Liver transplant is necessary
Common Deadly Mushrooms

Death cap
-A. *phalloides*,
-native to Europe but found widespread

Destroying angels
- A. *verosa*

Fool’s mushroom
- A. *verna*
Recent Local Fatal Cases

In 2014, 5 severe hospitalized cases for local residents:

• 3 Destroying angel
• 1 Fools’ mushroom (China)
• 1 Death cap (S. Africa)

1 dead, 1 required liver transplant
Erroneous Folklore
(from Wikipedia)

• Poisonous mushrooms are brightly colored
• Poisonous mushrooms have a pointed cap, edible ones have a flat, rounded cap
• Poisonous mushrooms blacken silver
• Poisonous mushrooms taste bad
• Poisonous mushrooms will turn rice red when boiled
• Mushrooms are safe if thoroughly cooked
• ............
Suspected Poisons (II)

8 victims, 1 died (2 days after consumption)

Quick onset: 10 min
Vomit, sore throat, headache, difficult breathing, body paralyze
## Analytical Results

<table>
<thead>
<tr>
<th>Toxins</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>K</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amanitin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>β-Amanitin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>γ-Amanitin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phallacidin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Muscarine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

DNA analysis showed it was *Russula subnigricans*
Russula Subnigricans

Literature information:
Contains trace or no amanitins and phallicidins

Cycloprop-2-ene carboxylic acid is the major component to cause toxic effects
Cycloprop-2-ene carboxylic acid

Almost Impossible to detect in vitro by instruments

- A highly strained 3-carbon ring natural compound → Rhabdomyolysis
- Breakdown of skeletal striated muscles
- Substances enter bloodstream → tea coloured urine in 12 -14 hrs
- Kidney failure, collapsed
- Matched with WHO’s descriptions

Mutually Beneficial

• Analytical report:
  Case I: *Amanita exitialis* containing $\alpha$-, $\beta$-, $\gamma$-amanitin & phallacidin
  Case II: *Russula Subnigricans*

• WHO set up preventive measures, eg. educational programmes to teach villagers on wild mushrooms

• Possibility of technology transfer or technical training

• GL: Invaluable lesson to mushroom toxins
Authentication of Botulinum Toxin (BoNT or Botox) in Pharmaceuticals
Fake Botox Products

News › China › Policies & Politics

Beware of fake Botox injections, China’s drugs watchdog warns

Warning comes following several people’s hospitalisation after receiving dodgy beauty treatment

PUBLISHED: Friday, 03 June, 2016, 1:08pm
UPDATED: Friday, 03 June, 2016, 11:17pm

Latest news on 3 Jun 2016 about extensive use of fake botox in China
Introduction: Botox

• Produced by *Clostridium Botulinum*
• Seven botox serotypes (A-G)
• Botox causes **botulism** in human by inhibiting the release of a neurochemical transmitter, acetylcholine (Ach) leads to nerve blockade and muscle disorders
• Onset: 18 – 36 hrs
• Symptoms: Blurred vision, drooping eyelids, slurred speech, difficulty swallowing, muscle weakness
• Infection via wounds, contaminated food, spores
3D Structure of Botox

- A protein containing about 1,300 amino acids
- MW of Botox A: 150kDa (Heavy chain ~100kDa + a light chain ~50kDa via a disulfide bridge)
## Biochemical Weapon Choice

Highly toxic: $LD_{50} \sim 0.0002 \text{ mg/kg}$; estimated 1 g could kill 1 million people (an ideal biochemical weapon)

<table>
<thead>
<tr>
<th>AGENT</th>
<th>$LD_{50}$ (mg/kg)</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botox</td>
<td>0.0002</td>
<td>Bacterium</td>
</tr>
<tr>
<td>Tetanus Toxin</td>
<td>0.002</td>
<td>Bacterium</td>
</tr>
<tr>
<td>Ricin</td>
<td>3</td>
<td>Castor Bean</td>
</tr>
<tr>
<td>VX</td>
<td>15</td>
<td>Chemical Agent</td>
</tr>
<tr>
<td>Soman (GD)</td>
<td>64</td>
<td>Chemical Agent</td>
</tr>
<tr>
<td>Sarin</td>
<td>100</td>
<td>Chemical Agent</td>
</tr>
</tbody>
</table>
Action of Botox

Emerging Infectious Diseases (2005) 11: 1578-1583
Clinical Applications

• Diluted Botox preparations have been applied to treat different symptoms since late 1960s:
  Ophthalmology: blepharospasm, strabismus
  Upper motor neuron syndrome: spasticity
  Excessive sweating
  Chronic migraine

• Came to cosmetic industry, relieve facial wrinkles. Botox A was first used to remove glabellar frown lines in USA in 1989
Beauty Business (wonder drug)

- USFDA approved Botox A (BOTOX®) in April 2002 for removal of facial wrinkles
- Projected a global market at > USD 4 billions in 2018
- Also registered as pharmaceutical products in Hong Kong
### Registered Products

<table>
<thead>
<tr>
<th>BOTOX A PRODUCTS</th>
<th>COUNTRY OF ORIGIN</th>
<th>DOSAGE</th>
<th>EXCIPIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botox</td>
<td>USA</td>
<td>100 IU</td>
<td>HSA (0.5 mg) NaCl (0.9 mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 IU</td>
<td></td>
</tr>
<tr>
<td>Xeomin</td>
<td>Germany</td>
<td>50 IU</td>
<td>HSA (0.5 mg) Sucrose (4.7 mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 IU</td>
<td></td>
</tr>
<tr>
<td>Dysport</td>
<td>UK</td>
<td>300 IU</td>
<td>HSA (0.5 mg) Lactose (2.5 mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 IU</td>
<td></td>
</tr>
<tr>
<td>Siax</td>
<td>Korea</td>
<td>100 IU</td>
<td>HSA (0.5 mg) NaCl (0.9 mg)</td>
</tr>
<tr>
<td>BTXA</td>
<td>China</td>
<td>50 IU</td>
<td>Gelatin (5 mg) Dextran (25 mg) Sucrose (25 mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 IU</td>
<td></td>
</tr>
</tbody>
</table>

By law, registered botox should be distributed to use on human by a licensed medical practitioner
Analysis of Botox

• Product (Injection formulation) compliance tests listed in pharmacopeias (eg. BP, USP) or AOAC Official Method (977.26) for consistency, stability, safety and efficacy using *in vivo* bioassay, however ....

Large number of mice
4-day monitoring for symptoms
Inhumane
Pressure from animal welfare organizations
Instrument Analysis?

- **Intrinsic issue**: Large MW (150kDa)

- **Extrinsic issue**: ultra-trace amount (pg to fg); severe interference from stabilizing proteins (mg)

- **Health issue**: extremely toxic, specialized laboratory facility required

- Chemical methods not easy; limited literature info
  - NIBSC – ELISA
  - USCDC – LC-MS/MS with sophisticated reagents, US patented
Our Approach

(a) Use modified SNAP-25 protein (substrate), 187-203 (17 aa)

(b) Add to botox A in buffer medium

(c) Botox A cleaves the substrate forming two fragments

(d) Confirmation of fragments by MALDI-TOF, LC-MS/MS or LC-TOFMS

(e) Indirect detection of Botox A
Endopep-MS Method

---D-M-G-N-E^{170}-I-D-T-Q-N^{175}-R-Q-I-D-R^{180}-I-M-E-K-A^{185}

-D-S-N-K-T^{190}-R-I-D-E-A^{195}-N-Q^{197}\textcolor{blue}{R^{198}}-A-T^{200}-K-M-L-G-S^{205}-G

187 203

Botox A

SNKTRIDEANQ^{197}\textcolor{blue}{R^{198}}ATKML (modified substrate)

17 aa

SNKTRIDEANQ^{197} (N-terminal product)

11 aa

R^{198}ATKML (C-terminal product)

6 aa

LC-MS/MS

MALDI-TOF
Designed Experiments

1. **Capture**: separation of botox A from matrix
2. Cleave disulfide bridge to free Lc of botox A
3. Optimize *in vitro* proteolytic conditions of Lc on the modified substrate: buffer, duration, temp, amount of substrate used, possible interference study, etc.
4. Optimize LC columns for good separation
5. Detection: MALDI-TOF and LC-MS/MS systems
6. Source out biotech companies to synthesize substrate and substrate fragments
Capture Technique

- Using polyclonal Ab coated magnetic beads
- Effective removal of matrices interference
- Improved capturing botox A
Substrate standards

SNKTRIDEANQ\textsuperscript{197} - R\textsuperscript{198}ATKML

R\textsuperscript{198}ATKML (C-terminal product)
SNKTRIDEANQ\textsuperscript{197} (N-terminal product)
MRM chromatogram for SNAP-25^{187-203} substrate and the corresponding cleavage product standards using C18 column.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Q1 (m/z) [M+2H]^2+</th>
<th>Q3 (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNKTRIDEANQ^{197-203} (\text{SNAP-25}^{187-203})</td>
<td>1009.0</td>
<td>226,129, 84</td>
</tr>
<tr>
<td>SNKTRIDEANQ^{197} (N-terminal product)</td>
<td>659.7</td>
<td>226,130, 84</td>
</tr>
<tr>
<td>(R^{198}\text{ATKML)} (C-terminal product)</td>
<td>359.7</td>
<td>183, 129, 84</td>
</tr>
</tbody>
</table>
## Optimization for Real Samples

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>HEPES (M)</th>
<th>DTT (mM)</th>
<th>ZnCl₂ (mM)</th>
<th>BSA (mg/mL)</th>
<th>Substrate (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>25</td>
<td>0.025</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>25</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
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<tr>
<td>3</td>
<td>0.05</td>
<td>25</td>
<td>2.5</td>
<td>1</td>
<td>1</td>
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<tr>
<td>4</td>
<td>0.05</td>
<td>25</td>
<td>25</td>
<td>1</td>
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<td>6</td>
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<td>2.5</td>
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<td>0.05</td>
<td>1.5</td>
<td>2.5</td>
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<td>9</td>
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<td>5</td>
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<td>1</td>
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<td>10</td>
<td>0.05</td>
<td>1.5</td>
<td>0.75</td>
<td>1</td>
<td>1</td>
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<tr>
<td>11</td>
<td>0.05</td>
<td>5</td>
<td>2.5</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

HEPES: buffer for all reactions; DTT: cleave S-S; ZnCl₂: cleave substrate; BSA: major excipient
Reaction time investigation
Cleavage products produced by real sample BXTA (Botox A 100 IU)

- **R₁⁹⁸ATKML**
  (C-terminal product)

- **SNKTRIDEANQ₁⁹⁷**
  (N-terminal product)

- **SNKTRIDEANQ₁⁹⁷-R₁⁹⁸ATKML**

**Botox A Sample**
SYNAPT G2 Q-TOFMS

Substrate standards

SNKTRIDEAQ^{197} (N-terminal product)

R^{198}ATKML (C-terminal product)

Cleavage products in pharmaceutical sample

Botox A detected
Confirmation (Q-TOF MS/MS)

Control

**R<sup>198</sup>ATKML (C-terminal product)**

Analyte: PATKML

Sample

**R<sup>198</sup>ATKML (C-terminal product)**

Control

**SNKTRIDEANQ<sup>197</sup> (N-terminal product)**

Analyte: SNKTRIDEANQ

Sample

**SNKTRIDEANQ<sup>197</sup> (N-terminal product)**
Calibration of Two Fragments

For quantitative purpose

Linear range = 10 – 100 IU

\[ \text{[RL-6]}^2+ \]
\[ (359.7/84.0) \]

\[ \text{R} = 0.9977 \]

\[ \text{[SQ-11]}^2+ \]
\[ (659.3/84.0) \]

\[ \text{R} = 0.9969 \]
What We Achieved

1. Positive identification of Botox A in all registered pharmaceutical products
2. Linear calibration curves established for quantitation
3. Reporting limit = 10 IU
4. Analytical service provided to law enforcement authorities in autumn 2016
5. Possibly replace animal test
6. Authenticate botox A in pharmaceutical samples
Future Work

1. Extend to measure botox A and other serotypes in food matrices to investigate possible botulism

2. Develop a Direct quantitative method by looking at signature peptide marker in botox A (in progress)
THANK YOU