Analytical Challenges in Veterinary Toxicology: Bromethalin

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CAHFS Toxicology
So you want to poison a rodent?

- Numerous chemical rodenticides are commercially available
- Anticoagulant rodenticides (e.g. d-Con) have been among the most popular over the last couple of decades
- In 2008, the EPA decided that some of these formulations pose an unreasonable risk to children, pets, and wildlife
- The EPA recently made a deal with Reckitt Benckiser to withdraw 12 d-Con products from the market
- So what’s a good alternative?
Bromethalin

- A substituted diphenylamine which causes CNS depression leading to paralysis and death
- Rapidly metabolized to desmethylbromethalin (the toxic agent)
- LD$_{50}$s in the 0.5 – 15 mg/kg range
- Available under trade names such as Tomcat, Assault, Rampage, Top Gun
- First released in 1985, but not as popular as anticoagulants (or at least so far)
Bromethalin poisonings in pets appear to be on the rise

From article in JAVMA News, July 1, 2014, https://www.avma.org/News/JAVMANews/Pages/140715m.aspx
CAHFS interest in Bromethalin

- As a veterinary toxicology facility, we are involved in diagnosing poisonings in animals
- Confirmation of exposure is best done via detection of the toxicant in the tissue or body fluid of affected animals
- Common tissue types analyzed include liver, fat, brain, muscle, etc.
- Several years ago, we had an animal which was suspected to have died of bromethalin poisoning
- Needed a method for bromethalin analysis in tissue
Structures

Bromethalin
(m.w. = 577.8)

Desmethylbromethalin
(m.w. = 563.9)
Bromethalin in the literature

- Search PubMed for “Bromethalin analysis” and all of seven publications show up, four co-authored by Dr. David Dorman

- Bromethalin and desmethylbromethalin have been detected in tissues from exposed animals and humans

- Bromethalin levels in dog tissue ranged from 10 ppb in brain to 1800 ppb in fat

- Desmethylbromethalin (DMB) ranged from 60 ppb in liver to 144 ppb in fat
Analytical Methods

- Methods have included TLC, GC-ECD, GC-NICI-MS, HPLC-DAD, and HPLC-APCI/MS
  - Bromethalin shows a broad UV absorbance band from 300 – 415 nm
  - APCI/MS work did not detect intact bromethalin, instead getting ions of varying m/z related to [M-Br]⁻, [M-HBr-NO2]⁻, etc.
- Bromethalin is highly photoreactive
  - According to Dr. Dorman’s data, 2 – 3 hours of irradiation by a 75-watt incandescent lamp caused a decrease of 50% response in a bromethalin standard solution analyzed by GC-ECD.
  - After 7 – 8 hours of irradiation, bromethalin was not detected
  - Desmethylbromethalin was only minimally photoreactive, with a 10% loss of response from 8 hours of irradiation
Bromethalin by LC-MS/MS: Early efforts at CAHFS

- After looking at the literature, we decided to try negative ion electrospray LC-MS/MS
- We followed the typical development procedure:
  - Infuse a standard solution into the MS source
  - Develop chromatographic conditions
  - Test extraction methods
Bromethalin standard, infusion spectrum, negative ion mode, ion trap

$\text{m/z } 560/562/564/566$

$\text{m/z } 465/467/469$
MS/MS of $m/z$ 562

$m/z$ 402

$m/z$ 404
Initial method (for liver/brain/fat)

- Homogenize 1 g. sample with 20 mL of 5% ethanol in ethyl acetate. Take a 5 mL aliquot, evaporate dry, reconstitute in 0.5 mL methanol
- Use 50 x 2.1mm, 1.7 µm Zorbax SB-C18, isocratic at 90% methanol, 10% of 0.1% formic acid in water
- Analyze on Thermo LTQ, with full scan negative ion MS/MS of m/z 562 and m/z 564
50 ppb Bromethalin spike
Issues regarding this approach

- 50 ppb was barely detectible. We wanted 10 ppb or less.
- Mass spectral information remained ambiguous – were we seeing bromethalin losing a methyl group in the ion source or were we seeing desmethylbromethalin or…..?
- Analysis of tissue samples from submitted animals did give positive results in a few cases
Finally….

- Toronto Research Chemicals put a desmethylbromethalin standard out on the market in early 2013
- Infusion spectrum showed the same 562/564/566/568 ions obtained from our certified bromethalin standard
- Retention time was identical to that obtained from our certified bromethalin standard
- It was clear that we were seeing desmethylbromethalin in our bromethalin standard
- Major difference between the two was sensitivity – we were apparently far more sensitive to the desmethylbromethalin standard than the bromethalin standard
So what was the issue?

- We ran the bromethalin standard several different ways:
  - A 1 mg/mL bromethalin standard run on the Exactive under high resolution/full scan settings showed over 40 peaks in the 400 – 600 amu range with Br$_2$ or Br$_3$ isotope patterns, none which appeared to be intact bromethalin
  - Optimized a method on a triple quad, looking for product ions of 562 and 576 and extended HPLC conditions. Still did not see anything that looked like intact bromethalin in the bromethalin standard
  - Finally ran the 1 mg/mL bromethalin standard in full scan mode with a UV detector plumbed in ahead of the mass spec…
1 mg/mL Bromethalin standard

Full scan, m/z 564

Full scan, m/z 574

DAD @ 360 nm
Best to go for DMB only

- Apparently, bromethalin does not ionize well at all by ESI
- DMB is the active metabolite and has been detected in tissue from dosed animals
- Bromethalin is photoreactive, DMB is not.
Method for DMB in tissue

- Extraction method is the same as mentioned before
- HPLC conditions:
  - 100 x 2.1, 1.7 µm Zorbax Eclipse Plus C18
  - Mobile phases: 0.1% formic in H2O and 0.1% formic in ACN
  - Gradient starts at 20% organic, holds for one minute, goes to 95% at 9 min., holds for 4, then back to initial conditions and 4 min equilibration
- MS conditions:
  - Negative ion electrospray ("Jet Stream") on an Agilent 6460
  - MRM of m/z 562 -> 278, CE = 25 and 562 -> 254, CE = 35
250 ppt DMB std (MRM)

m/z 562 → 254
Validation

- Used fat as a matrix
- Extracted calibrators at 1, 5, 10, 25, 50 ppb
- 6 spikes each at 1, 5, and 50 ppb
- MDL = 0.35 ppb

<table>
<thead>
<tr>
<th>Spike Level (n=6)</th>
<th>Mean % Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ppb</td>
<td>110</td>
<td>11</td>
</tr>
<tr>
<td>5.0 ppb</td>
<td>110</td>
<td>15</td>
</tr>
<tr>
<td>50 ppb</td>
<td>105</td>
<td>24</td>
</tr>
</tbody>
</table>
1 ppb spike in fat

$\text{ESI MRM Frag}=135.0V\text{ CID@** (562.0} \rightarrow 254.0)\text{ 080813-11.d}$

$m/z\ 562 \rightarrow 254$

$m/z\ 562 \rightarrow 278$
Incurred DMB in adipose tissue from a fox

\[ m/z \, 562 \rightarrow 264 \]

\[ m/z \, 562 \rightarrow 278 \]
Conclusions

- Analysis for desmethylbromethalin by LC-ESI/MS/MS is an effective method of establishing bromethalin exposure in affected animals.
- The method is sensitive, specific, and efficient.
- LC-MS using electrospray ionization is ineffective for analysis of intact bromethalin at ppb levels.