ETHYLENE GLYCOL RAPID METHODS OF DETECTION

by

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Abstract

Every year thousands of domestic animals are poisoned by ethylene glycol. Exposure is normally orally, but may be dermal, and poisonings are usually accidental and not malicious. Antifreeze, overwhelmingly the source of the ethylene glycol poisoning, is responsible for over 99% of reported cases. Storage, handling and proper disposal of ethylene glycol is extremely important in limiting access to this deadly product.

Ethylene glycol exposures were involved in 1737 calls made to the American Society for the Prevention of Cruelty to Animals call center between 2006 and 2011. Dogs were involved in approximately 87% of exposures and cats in 13%. There were no seasonal or breed patterns. The most common clinical signs reported were neurological and gastrointestinal for both cats and dogs. Urinary calcium oxalate crystals were reported in 28.6% of exposed cats, and 21% of dogs.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was used to detect calcium oxalate crystals in wax-mounted kidneys from twenty total cases, ten of which were suspected ethylene glycol poisoning submitted to the Kansas State Veterinary Diagnostic Laboratory, and ten samples deemed negative by a pathologist using light microscopy. Pure calcium oxalate monohydrate was used as a reference, and a unique absorption peak was detected between wavenumbers 1290 cm^{-1} and 1320 cm^{-1}. The drying of kidney tissues resulted in increased sensitivity for calcium oxalate. Crystal detection by the ATR-FTIR was compared to light microscopy. Bi-fringence of crystals allowed microscopic detection, but the ATR-FTIR specificity for the test was 100%, and sensitivity was 80% compared to traditional microscopy for ca-oxalate crystal identification.

ATR-FTIR was also used to detect un-metabolized ethylene glycol in vomitus using wavenumbers 1084 cm^{-1}, 1039 cm^{-1}, and 882 cm^{-1}, but ethylene glycol was not detectable. Ethylene glycol concentrations in samples were much too low to be detected as ethylene glycol on the ATR-FTIR, as the limit of detection was not distinguishable until 5000 ppm using a serial dilution. These methods presented simple, reliable, quick, sensitive, stable, and highly adaptable tests for detection, diagnosis and treatment of ethylene glycol poisoning.

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Chapter 1 - Introduction and Literature Review

Introduction

As accidental and malicious poisonings play an important role in animal health, the ease of availability of products containing ethylene glycol leading to exposure and subsequent poisoning for both domesticated animals and wildlife is a major concern. Many times products containing ethylene glycol are not stored, handled, or disposed of in a safe and consistent manner. Ethylene glycol is the primary constituent of household chemicals such as automotive antifreeze, and hydraulic brake fluid, and is being used as a solvent in many industrial processes. It is a clear, colorless, odorless, and viscous fluid with a sweet taste that animals readily ingest. Ethylene glycol when manufactured as antifreeze may appear colored due to the addition of added pigments like fluorescein. The addition of fluorescein aids in the detection of automotive leaks. Dogs and cats are the most frequently involved species in poisoning cases (Leth, 2005).

Following ingestion of ethylene glycol there is rapid absorption from the digestive tract. Ethylene glycol converts to glycol-aldehydes and organic acids, including oxalic acid, in the liver. Oxalic acid reacts with calcium in the blood and tissues to form insoluble calcium oxalate crystals deposited in tissues, particularly renal tissue, and excreted into the urine. Far less data exists for detection of ethylene glycol prior to metabolization. Rapid detection and confirmation of calcium oxalate and ethylene glycol is the key to a diagnosis and life-saving treatment for suspected animal poisonings (Terlinski, 1981).

Epidemiological data from the American Society for the Prevention of Cruelty to Animals (ASPCA) 2006-2011 assessed in Chapter 2 gives insight into the most common species and breeds involved in poisonings. Seasonal and geographical data, exposure routes, and symptoms, along with predictions and limitations of examined data were discussed. Comparisons to already published data will be used to support or contradict these findings. The ultimate goal would be to be able to give owner's useful knowledge to prevent/decrease exposures to the common household chemical ethylene glycol and promote animal health.

Literature Review

Characteristics

Ethylene glycol is formed by mixing ethylene oxide and water:

$$C_2H_4O + H_2O \rightarrow HO–CH_2CH_2–OH$$

Ethylene glycol has a density of 1.1 g/cm³ which is near that of water; it is miscible in water and soluble in most organic solvents. The reaction is catalyzed using either acids or bases or at a neutral pH with an elevated temperature (Rebsdat, 2005). Ethylene glycol has a low vapor pressure and poor skin absorption so most poisonings occurred via ingestion. Ethylene glycol has a flash point > 100°F that makes it a combustible material. Ethylene glycol disrupts hydrogen bonding when dissolved in water as it is in antifreeze. Pure ethylene glycol freezes at about −12 °C (10.4 °F), but when mixed with water, the mixture does not readily crystallize, and, therefore, the freezing point of the mixture is depressed. Specifically, a mixture of 60% ethylene glycol and 40% water freezes at −45 °C (Rebsdat, 2005). Ethylene glycol is a central nervous system depressant similar to that of alcohol. Table 1-1 outlines the physical properties and chemical identity of ethylene glycol.

Table 1-1: Physical and Chemical Properties of Ethylene Glycol

Property	Ethylene Glycol
Chemical formula	$C_2H_6O_2$
Molecular weight	62.07
Color	Clear, colorless
Physical State	liquid
Melting point	- 12.69 ^{o} C
Boiling point	197.3 ^{o} C
Density: at 20 ^{o} C	1.1135
Odor	Odorless
Solubility:	
Water at 20 ^{o} C	Miscible with water
Organic Solvents	Soluble in lower aliphatic alcohols, glycerol, acetic acid, acetone; slightly soluble in ether; practically insoluble in benzene, chlorinated hydrocarbons, petroleum ether, oils.
Vapor pressure at 25 ^{o} C	0.089 mm Hg
Auto-ignition temperature	398 ^{o} C
Flashpoint	127 ^{o} C

Stages of Poisoning

Ethylene glycol poisoning typically occurs in three stages: A central nervous system (CNS) depression phase, a cardiopulmonary phase and lastly a renal toxicity phase. These stages may have some overlap and do not adhere to strict guidelines as they are time-dose dependent. In the first stage, the CNS becomes depressed, and this stage lasts for up to twelve hours. The subject appears "drunk". Between four and twelve hours after ingestion, the glycol-aldehyde forms and clinical signs including ataxia, polyuria, polydipsia, seizures, unconsciousness, edema, nausea, and vomiting may occur(Gupta, 2012).

An osmolal gap or an anion gap may be detected in the first stage before much of the ethylene glycol metabolizes. However, some animals have normal osmolal and anion gaps and have no signs of metabolic acidosis. But, as the ethylene glycol is metabolized the osmolal gap should decrease and the anion gap emerges. Dogs may appear to recover from CNS signs at about 12 hours after ingestion, but they have not recovered from the toxic effects (Gupta, 2012).

Stage two clinical signs usually appear twelve to twenty-four hours after ingestion and include tachycardia, tachypnea, hypertension and hypotension. In addition to the previous symptoms, conditions such as edema, pneumonitis, cardiac failure and shock may result. Oxalic crystal formation and deposition will begin to cause tissue injury and hypocalcemia. Stage two is when most deaths occur. In stage two blood tests can detect hypocalcemia.

Stage three, is characterized by oliguric renal failure, and distinguished by proteinuria, hematuria, crystalluria and increased blood urea nitrogen (BUN) and creatinine. The calcium oxalate crystals responsible for the blockages in the kidney are typical of stage three, but may form as early as stage one. Renal failure in cats usually develops between 12 – 24 hours, and between 36 – 72 hours in dogs (Gupta, 2012).

Diagnosis

Many non-specific clinical signs and conditions arise from ethylene glycol ingestion and poisoning, making it difficult to diagnose. The most common signs of ethylene glycol poisoning mimic other CNS diseases, trauma, diabetes or gastroenteritis. Nearly the only way to rapidly diagnose ethylene glycol is by witnessing consumption. Laboratory testing will be beneficial one to two hours after consumption allowing enough time for ethylene glycol to show up in the serum and urine (Peterson, 2013).

In the case of undetected consumption, a diagnosis would come from a combination of animal health history, investigation into access to chemicals, a timeline of symptoms, and a physical examination of the animal along with laboratory testing. But, many times laboratory testing comes too late for survival purposes and is used only as a means of resolution. Timely treatment is the difference in life or in death in ethylene glycol poisoning cases.

Detection of ca-oxalate crystals in urine and kidney serve as an invaluable tool for detection, confirmation, and treatment of ethylene glycol poisoning. Specifically testing serum or urine is a quick means of early detection. Laboratory blood tests detect osmolal and anion gaps due to the metabolic acidosis and accumulation of glycolic acid, but the gap is not always detectable. Hours after consumption the patient may become hypo-calcemic due to losing calcium in the formation of ca-oxalate crystals (Gupta, 2012).

Sadly, for most animals the testing of submitted samples is usually post-mortem and is not for diagnosis of disease, but for confirmation of a cause of death. Kidneys submitted for histological examination of the tubules for ca-oxalate crystal detection as well as urine and blood are considered suitable samples for submission (Peterson, 2013).

Oral Intake

The minimum lethal dose of ethylene glycol is a relatively small amount. The undiluted minimum dose for cats is 1.5 mL/kg of body weight (Milles, 1946), for dogs it is 6.6 mL/kg (Kersting, 1966). Most antifreeze is approximately 50% ethylene glycol and about 50% water.

Initially, about half the ethylene glycol is excreted unchanged through the kidney. The remainder is oxidized by the liver and kidney and results in toxic metabolites that lead to

metabolic acidosis and renal tubular damage. First, the enzyme alcohol dehydrogenase in the presence of ethylene glycol forms a glycol-aldehyde that rapidly metabolizes to glycolic acid. The glycolic acid is further oxidized to glyoxylic acid. The glycolic acid accumulates as the formation of glyoxylic acid is a rate-limiting step. The overabundance of glycolic acid results in acidosis. The glyoxylic acid metabolizes to formic acid, carbon dioxide, glycine, serine, and oxalate. Oxalate unable to be further metabolized, combines with calcium in the body and forms ca-oxalate crystals. These crystals are microscopically observed as early as 3 and 6 hours after ingestion in the cat and dog, respectively (Dial, 1994a, b) and then can be detected via Attenuated Total Reflectance Fourier Transform Infrared Spectrometer (ATR-FTIR).

Dermal Absorption

Acute exposure studies reveal ethylene glycol on skin has a rate of absorption so slow that excretion and metabolization pathways are able to handle the small amount absorbed, no metabolites accumulate and therefore poisoning does not occur (Sun, 1995). In a dermal study, ethylene glycol was tested on mouse skin with steady state reached at 3-5.5 hours, and the absorption was quite low at 0.52 mg/cm²/h. The 50% solution of ethylene glycol was 0.22 mg/cm²/h (Sun, 1995). Concurrent testing on human skin revealed that human skin absorption was about 30 times slower than on mouse skin; diluted ethylene glycol to a 50/50 solution with water as found in most antifreeze products resulted in an absorption rate that decreased by half. Due to the negligible amount absorbed, that study concluded that dermal exposure posed no real threat to animal health and safety.

Crystals

Calcium oxalate crystals commonly found in tissues and urine are part of the clinical course and diagnostic detection of ethylene glycol poisoning. The absence of crystals does not rule out poisoning (Vale, 1979). If a large amount of ethylene glycol is ingested the metabolism moves toward a zero order elimination that results in a build-up of glycolic acid, leading to death before oxalic acid forms (Peterson, 2013). Oxalic acid, when combined with calcium, forms

calcium oxalate mono-hydrate (Jacobsen, 1986). Deaths that occur quickly result in little to non-existent crystal formation. Crystal formation is necessary for detection microscopically and for detection on the ATR-FTIR. Several crystals found in urine, but not recognized as calcium oxalate include: struvite, cysteine, ammonium urate, xanthine, and silicum dioxide (Hesse, 1990). The most common crystals found in both tissues and urine that indicate ethylene glycol poisoning are calcium oxalate di-hydrate (weddellite) and calcium oxalate mono-hydrate (whewellite) (Jacobsen, 1988). Calcium oxalate di-hydrate crystals are often dumb-bell shaped and poorly bi-fringent under polarized light. Di-hydrate crystals are also found to be envelope shaped a.k.a. Maltese cross (Connally, 1996) and highly bi-fringent. This form is often found using light microscopy. Calcium oxalate crystals are also found to be hemp seed shaped, although it is rare. Calcium oxalate mono-hydrate crystals are also, at times, found to be dumbbell shaped (Jacobsen, 1988).

The calcium oxalate mono-hydrate crystal shaped like a six-sided prism is doubly refractive. The four-sided crystal was previously thought to be a hippurate-like crystal formed from hippuric acid, but X-ray diffraction shows that it was in fact calcium oxalate monohydrate crystals, and very useful in detection of poisoning. X-ray diffraction is a useful tool for identifying calcium oxalate crystals in concentrations greater than 3% (Thrall, 1985). In urine, calcium oxalate di-hydrate crystals underwent dissolution and re-crystallization to the mono-hydrate form at about 24 hours (Thrall, 1985). The mono-hydrate form is highly specific to ethylene glycol poisoning (Gupta, 2012).

In a study of a combined total of fifty cats and dogs poisoned with ethylene glycol, only 18% of the urine samples contained calcium oxalate monohydrate crystals (Thrall, 1985). This study supported the notion that not all poisonings result in crystal formation or detectable crystal formation, and that crystals can form for many reasons that are not necessarily ethylene glycol poisoning. Urinary crystals in the shape of needles were often detected along with calcium oxalate crystals during ethylene glycol poisoning, but this shape of crystal lended itself more towards acidic urine and renal failure (Jacobsen, 1988).

Light microscopy is not always sufficient to identify crystals, as many crystals with the same apparent morphology are actually composed of different substances. Laboratory personnel must be aware of the many shapes the crystals form. In this way, FTIR eliminates the problem in crystal identification, as FTIR uses infrared absorption with unique peaks between 1330 and

1290 cm^{-1} for ca-oxalate crystal identification and peaks 1084 cm^{-1}, 1039 cm^{-1}, and 882 cm^{-1} for ethylene glycol detection.

Calcium oxalate crystals are found throughout the kidney, but are most often located in the tubules. In a study, kidney epithelial cells of the BSC-1 line were used to model the tubule. It seemed that these cells rapidly bound and internalized crystals of calcium oxalate mono-hydrate. Electron microscopy of the sites revealed that adherent crystal on the cells surface served as sites for aggregation of additional crystals (Leiske, 1997). This aggregation of crystals greatly helped in the diagnosis of ethylene glycol poisoning and provided an increased population of crystals in more localized areas.

Treatment

Once diagnosed with ethylene glycol poisoning, treatment needs to begin as soon as possible. Progression of the disease is largely dose dependent, but generally speaking treatment for dogs would ideally begin within five hours of exposure and treatment for cats would begin within about three hours for a good prognosis (Gupta, 2012). Depending upon how far the disease has progressed would depend upon the course of action to counteract the symptoms and damage to the body.

If the poisoning is caught early, the normal procedure involves giving sodium bicarbonate to counter-act the production of organic acids. Next, ethanol or fomepizole would be administered to prevent further metabolization of ethylene glycol. Fomepizole inhibits alcohol dehydrogenase (ADH) from propagating the glycolic acid metabolite being formed, while ethanol acts as a competitive antagonist to ethylene glycol which simply means ADH prefers ethanol over a reaction with ethylene glycol (Penumarthy, 1975). ADH is a group of enzymes that convert and break down toxic alcohols into metabolites.

Ethanol is a better choice when treating cats as they do not receive the same benefit that dogs do when using fomepizole. Cats require a much larger dose of fomepizole that can be very expensive and still not as effective as a treatment. Ethanol has the advantage of being inexpensive and effective for cats, but the cat must be very closely monitored as ethanol is hard to regulate, and it also compounds the CNS depressive effects present from ingesting the ethylene glycol initially (Gupta, 2012). The use of ethanol was not considered an officially

approved method for dealing with ethylene glycol poisoning by the Food and Drug Administration (FDA) (ASTDR, 2010) in human cases of poisoning. In 1997, the FDA approved the use of Fomepizole as a treatment for ethylene glycol poisoning in humans and a few years later the criteria for use of Fomepizole in animals was developed by the American Academy of Clinical Toxicology (ASTDR, 2010).

Once a significant amount of ethylene glycol has been metabolized, and clinical signs including azotemia and oliguric renal failure occur, the use of Fomepizole or ethanol is of little value. Hemodialysis could be used and supportive care initiated as clinical signs arise. Hemodialysis is useful in removing ethylene glycol and glycolic acid and any other waste products that are already present in the body. Hemodialysis will not prevent or reverse kidney failure (Cowgill, 2000). Fluid therapy would be utilized to increase urine production for elimination of waste and to decrease dehydration (Ross, 2011).

Other Testing Available

Plasma ethylene glycol tests

Specific tests for detection of ethylene glycol in blood or plasma are available as colorimetric comparisons and as semi-quantitative strip tests. Blood must be drawn in an EDTA or heparinized tube or another compatible anti-coagulant, and then spun down before the plasma sample is extracted. These test strips and colorimetric comparisons work by visually comparing a color detected after the plasma sample is placed in a reservoir on the test strip or extracted and then compared with known colors at given concentrations on the test strip or colorimetric extractions of a positive control. The lethal concentration of ethylene glycol in plasma in dogs and cats is 50 mg/dl and 20 mg/dl, respectively (PRNEGTK, 2009).

These tests are portable, rapid, low cost, and easy enough that non-specialized personnel should be able to accurately perform the testing. These tests should be able to detect ethylene glycol within two hours of ingestion. Some kits can detect ethylene glycol as early as 30 minutes after ingestion, but only up to fourteen hours after ingestion (KEGT, 2009). Many tests have time limitations that need to be taken into account when

sampling, and these kits have expiration dates that must be adhered to ensure the integrity of the test along with refrigeration requirements.

Colorimetric testing and test strips have some limitations including interferences that may be inherent in the test or introduced in the matrix of the sample, such as propylene glycol or glycerol(Gupta, 2012). There can always be operator error or differences in operator determination because the results are interpreted visually and not tested quantitatively. Samples drawn outside of the window of detection of the ethylene glycol in the blood might be unsuitable. Test strips and kits may be expired or not handled in the required manner causing reagents to deteriorate (KEGT, 2009).

Miscellaneous Tests

Sodium fluorescein is added to anti-freeze as a colorant to be able to detect leaks if they should arise in the radiator of vehicles or if a spill occurs. Sodium fluorescein fluoresces under a wood's lamp. One early method of determining ethylene glycol consumption was to examine urine under a wood's lamp, and if it fluoresced then ethylene glycol may have been consumed (Winter, 1990).

There are many blood tests that indicate a diagnosis of ethylene glycol poisoning, and perhaps give an idea of patient prognosis. Blood tests including the testing of arterial blood gasses, blood glucose, and serum electrolytes to calculate anion gap (AG) and osmolal gap (OG). The following are the calculations required for determining the osmolal gap (Khajuria, 2005).

(1). CO = 2 x NA + 1.15 * GLU/18 + UN/2.8 : calculated osmolality
(2). OG = MO - CO : osmolal gap
(3). AG = (NA - HCO_3 – CL)

Measurements for line 1 calculations above include: calculated osmolality (CO), sodium (NA), glucose (GLU) and urea (UN). Line two calculations for osmolal gap include: measured osmolality (MO), which is measured on an osmometer in mol/kg, and calculated osmolarity (CO) which is a measure of mol/L. Line three calculations for

anion gap (AG) calculation included: sodium (NA), bicarbonate (HCO_3), and chloride (CL).

A situation where tests reveal a low pH, a high AG, normal ketone body value, normal glucose and a high OG are indicative of ethylene glycol poisoning (UIHC, 2014). The AG is merely the difference between measured cations and anions, and is used to indicate metabolic acidosis that can be a common finding in ethylene glycol poisoning. Metabolic acidosis is also a common finding in many other unrelated diseases; neither the finding of metabolic acidosis nor the lack of metabolic acidosis is definitive for ruling in or ruling out ethylene glycol poisoning on their own merit.

Other laboratory tests indirectly supporting a diagnosis of ethylene glycol are increased blood urea nitrogen (BUN) and creatinine, which are usually run in conjunction to evaluate kidney function. Calcium concentrations will decrease with formation of the calcium oxalate crystals. A urinalysis can reveal the formation of crystals, while an ultrasound can show if the liver and kidney were swollen. In cases of ethylene glycol poisoning ultrasounds may emit a denser echo due to the crystal formations that occur in the kidney and liver (Peterson, 2013).

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is useful for identifying unknown materials, looking for specific toxicants, determining quality or consistency of samples and detecting individual components of a mixture (TNIFTM, 2009). The attenuated total reflectance (ATR) -FTIR can be used to analyze organic and non-organic samples in a solid or liquid phase or adapted for testing in a gas phase (Rees, 2010). ATR is a sampling tool that directs an infrared beam into a higher refractive index substance, the beam is reflected, creates a wave, and the energy from that wave is either absorbed or reflected back to a detector (TNIFTM, 2009).

First, an infrared (IR) beam reflects off of a crystal with a high reflective index such as a diamond. This creates an electromagnetic wave known as an evanescent wave, which is absorbed by the sample and then the reflected radiation is returned to the detector. To create a spectrum, the IR radiation must move from a denser medium (diamond) to a thinner medium (sample). While a diamond containing instrument is more expensive, the use of a diamond

crystal allows for a wide range of samples to be tested as the diamond is highly reflective, inert, and ranks a ten on the Moh's hardness scale (West, 1986).

During the sample absorption various molecular vibrations are absorbed. Functional groups such as C-O, C-H, and N-H stretch bend and oscillate and along with their particular structure arrangement in the compound produce a unique and specific spectrum (Smith, 2011). Vibrational modes are active when the frequency of radiation co-incides with the frequency of the molecules oscillation (resonance) and their dipole moments change in the same direction. Measuring the amplitudes and frequencies of radiation absorption creates a spectrum used to identify functional groups and compounds (Griffiths, 2007). The spectrum gives a molecular absorption and transmission fingerprint.

There are many advantages of utilizing the ATR-FTIR over the older dispersive instrumentation. Advantages for the ATR-FTIR include better signal to noise (S/N) ratio, increased sensitivity, little to no sample preparation, non-destructive method of testing, speed of testing with simultaneous measurements, no external calibration is necessary and it is mechanically simple with only one moving part. Increasing the number of mirrors results in a stronger absorbance of the sample, but also increases the scattering of light and makes the light harder to quantify. One of the few disadvantages is that the instrument is susceptible to the effects of environmental conditions, so a background reading is required intermittently (Smith, 2011).

Specific advantages of the ATR-FTIR include the Fellgett advantage. Because the complete spectrum is collected all at once, the collection is rapid with many scans being averaged in a short period of time. Another benefit is the Jacquinot advantage that results from the averaging of the S/N ratio over the entire scan and allows for increased sensitivity. Lastly, the Connes advantage is due to the instrumentation having its own internal calibration with a known frequency scale that makes it very accurate and stable (Sun, 2009).

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Chapter 2 - Trends in Ethylene Glycol Poisoning 2006-2011

Introduction

The American Society for the Prevention of Cruelty to Animals (ASPCA) receives calls daily from animal owners seeking assistance for potential ethylene glycol poisoning of their animals. These animals have been exposed orally and dermally to household chemicals such as automotive antifreeze, inks, paints, wood stains and hydraulic brake fluid. Most of the poisoning cases occur accidentally in or around the home. Poisonings, at times, may be malicious. These data will examine the characteristics of the disease development as it was reported during the calls. Ethylene glycol poisoning is not the type of disease that spreads throughout a population or changes over time.

Dogs and cats are most frequently involved in reported poisoning cases due to the fact that they are the most prevalent companion animal species. Major causes of ethylene glycol intoxication are due to the mishandling of chemicals in regards to use, storage, and disposal. In addition, ethylene glycol can be administered in an effort to purposely poison an animal.

These examination of data will serve a case observation examining trends for suspected and observed ethylene glycol poisoning cases. Case observations assess the relationship between exposure and disease variables. These data do not necessarily represent the poisoning proportion in animals in the United States and Canada, but they are the best data available.

Methods

In this review, these data were compiled from 1737 calls that were received between January 1, 2006, and December 31, 2011, by the ASPCA concerning ethylene glycol exposures in animals. For each case, the following parameters were recorded: Case ID, Patient #, Group, Breed, Mixed, Age Range, Years Old, Kg Wt., Outcome, Total Patient Exposure, Exposure Certainty, Agent, Agent Certainty, Amount, Amount Certainty, Route, Sign Risk, Severity Risk, Assessed, Ingredient, Dose, Body System, Condition, Illness level, Opened, City, State, County,

Postal Code, and Geography. The parameter listed above for Opened refers to the date which the case was opened at the ASPCA call center.

After analysis, the cases were sorted by Year, Month, Group, Breed, Body System, Condition, Outcome and Location. Based upon the analysis of data, certain conclusions could be made about suspected trends and they could be substantiated. Information provided in other literature was used to support or dispute the data presented.

Results

Species involved

According to the data recorded from received calls (Table 2.1), domesticated pets were most frequently involved in exposures compared to livestock and wild animals. The combined total for help requests was 100% and included dogs at 87.6%, followed by cats 12.4% and then ferrets at 0.1% and other species (Horse, Pig, Raccoon, Unknown) accounted for 0.5%. In broader, but similarly compiled data from the ASPCA for all poisoning cases from 2002-2010, dogs accounted for 76% of reported calls and cats accounted for 13% (McLean, 2012).

Season and location

The bar chart (Figure 2.1) clearly demonstrates that the calls were consistent throughout the year. The volume of calls was highest in June (10.2%) and lowest in July (7%). During spring (March-May) the call volume rose gradually with a slight decline in the summer months following June. The calls also declined slightly during winter and fall. Figure 2.2 showed the data analysis with data points divided into the four seasons between the time periods of 2006 to 2011. The P value calculated was 0.684. Figure 2.2 calculations were a One-way ANOVA test with 95% Confidence Intervals for mean based on pooled standard deviation. The distribution of data among the seasons was not statistically different.

Calls were received from both the United States and Canada. The highest proportion of calls came from California (8.5%), followed by Pennsylvania (7.2 %) and New York (5.5%) as shown in Figure 2.3. Approximately 2.5% of the callers did not report any information on their

location either inside or outside of the fifty states, and nearly three percent of the callers resided in Canada.

In evaluation of species and location of calls received (Figure 2.4), four observations stand out. First, Wyoming had no calls made to the ASPCA poisoning hotline. Next, most calls for help came from the North Eastern portion of the United States. Third, Missouri was the only state to call on behalf of a non-domesticated animal, and lastly Louisiana was the only state with a higher proportion of calls for help for cats compared to that of dogs. However, the percentage of calls received by Louisiana was < 1.58% of the total calls received.

Cat and Dog Data Breakdown

The number of animal inquiries regarding ethylene glycol poisoning in dogs was 87%, while the number of poisoning or suspected poisoning cases in cats was 12%. The calls concerning other species made up less than 1% of the total and were not further examined in this data set.

The data examined for dog breeds included Labrador Retrievers with 20.8%, German Shepherds with 7.2%, followed by Golden Retrievers 6%, Beagles at 2.3%, and lastly Yorkshire Terriers at 1.8%. These numbers could be lower than what actually occurred because breed data was unspecified in 7.8% of the entries. The top five breeds represented approximately 38% of the total data.

The data examined for cats also included breeds: Abyssinian with 0.5%, Persians with 1.9%, Siamese with 2.3% and the Maincoon with 3.2%. The top five breeds according to the Cat Fancier's Association represented approximately 7.9% of the total data, while the domesticated cat accounted for 90.7% of the ethylene glycol poisoning calls.

Clinical Signs Reported for Dogs

The clinical signs for data cells left blank were not used in the following calculations. The total number examined was 2446 minus 1064 for a total of 1382 reported symptoms. Data from Table 2.2, revealed the most common reported clinical signs for ethylene glycol poisoning

and suspected poisoning cases in dogs were nervous clinical signs at 30.3%, followed by digestive at 22.5%, general clinical signs at 12.7%, urinary at 11.6% and metabolic clinical signs at 9.1%. The most common nervous clinical sign was ataxia at 36.5%, followed by lethargy at 23.4%. The most frequent digestive clinical sign in dogs was vomiting at 64.6%. General clinical signs included anorexia 15.9% and euthanasia was reported in 14.2%. Urinary clinical signs comprised crystalluria at 21.1% and polydipsia with 19.3%. Lastly, the most frequent metabolic clinical sign for dogs was azotemia at 21.4%. In Table 2.2, elevated creatinine was listed under urinary clinical signs and only accounted for 11.2%.

Clinical Signs Reported for Cats

The clinical signs for data cells left blank were not used in the following calculations. The total number examined was 491 minus 98 for a total of 393 reported clinical signs. Examined data from Table 2.3, revealed the most common reported clinical sign for ethylene glycol poisoning cases and suspected cases for cats was nervous clinical signs at 33.3%, followed by general clinical signs at 16.5%, digestive at 14.2%, metabolic clinical signs at 12.5% and urinary at 8.9%.

The most common nervous clinical sign was ataxia at 38.2%, followed by lethargy at 25.2%. The most frequent digestive clinical sign for the cat was vomiting at 60.7%. General clinical signs included anorexia 18.5% and vocalization and hypothermia both at 12.3%. Urinary clinical signs included formation of calcium oxalate crystals at 28.6%. Lastly, the most frequent metabolic clinical sign for cats was azotemia at 34.7%.

Exposure Routes

Figure 2.5 illustrates that about 92% of cases involved occurred by oral ingestion and 3.7% reported a combination of oral and dermal exposure. The dermal only route was reported 0.4% in these data. Other reported routes of exposure in these data included rectal, ocular,

inhalation, unknown, or not asked. Rectal, ocular and inhalation account for 0.5% of the exposure routes. Not asked and unknown were calculated at 1.1% and 2.3% respectively.

Discussion

These data were a case observation, and little to no information was available in terms of confirmation of poisoning or outcomes resulting in death. Follow-up calls were not made by members of the call center. All information was generally provided in an initial phone call and may have contained inconsistencies due to non-professionals relaying information(owners), generalized clinical signs, stress of the situation, unknown chemical or dose exposure, questions not asked by ASPCA members, inconsistent assessment by call center agents, and other factors. Dogs accounted for 87% of all cases, whereas cats were only 12% of the reported data. These data were supported by similar findings obtained in a study conducted in the United State from 1993 to 1994 listing dogs as 82.8% of reported poisoning cases and 13.6% of the cases were cats (Hornfeldt, 1997). Texas Poison Center data showed that 87% of poisonings occurred in dogs and 11% cats (Forrester and Stanley, 2004). In addition, the 27th annual report of the American Association of Poison Control Centers' (AAPCC) recorded that 90.6% of animal poison exposures were in dogs followed by cats with 8.2% (Bronstein, 2010).

In Brazil, Medeiros and his colleagues found in a retrospective study from 2002 to 2008 that dogs accounted for 86.1% and cats 13.9% of all cases (Medeiros, 2009). Dogs and cats accounted for 99.43% of pets involved this study due to the fact that dogs and cats were the most common pets in the United States. Livestock and wild animals were not monitored as frequently as pet animals. A possible explanation for differences between the number of dogs and cats involved in ethylene glycol poisoning could be related to the fact that cats tend to be more selective in their food choices refusing any food that does not smell appealing (Peterson, 2013). Cats do not prefer, and may lack the ability to detect the sweetness of sugar, as other animals, including dogs, do have this ability (Li, 2005). The higher incidence of poisoning could also be due to a dog's inquisitive nature (Peterson, 2013). Dog incidences of poisoning

were higher in spite of the fact that cats outnumber dogs in households 95.6 million to 83.3 million (APPA, 2014).

These ethylene glycol data seemed to be consistent throughout the year with each month having about 7% - 8% of the total calls received for the year with little variance year to year (Figures 2.1, 2.2). March and May increased to 9%, while the highest month was June with 10% of the calls. These data was similar to Forrester and Stanley (2004) but did not show the overall increase during summer months. The animal poison control center data revealed an increase in general poisonings in December around the holidays and an increase in the first month of spring. These ethylene glycol data showed an increase in the first month of spring, but no holiday increase in December. This could be due to the fact that the current data were only concerned with ethylene glycol and not all poisonings, which would probably have higher numbers in spring and summer with fertilizer, pesticides, insecticides being utilized, flowers blooming, and more access to outdoor activities (Peterson, 2013). The book, The Small Animal Toxicology, Third Edition (Peterson, 2013), relayed that ethylene glycol was seasonal with an increase in poisoning in the fall, winter and early spring. This statement was inconsistent with the analysis of these data.

The highest percentages of calls to the ASPCA were received from California, Pennsylvania and New York. In comparing these three states with the April 1, 2010, census, it is evident that higher populated states called the ASPCA more frequently. California had the highest population of any state at 12% (37,253,956 of 308,745,538) and represented 8.5% of the calls. Pennsylvania ranked number six with calls received and represented 4% (12,702,379 of 308,745,538) of the population and made 7.2% of the calls. Lastly, New York, which was ranked number three had 6% of the population (19,378,102 of 308,745,538) and represented 5.5% of the calls received (USC, 2014). Figure 1.4 visually emphasizes the direct correlation between the fifty states percentage of calls received and the species involved in ethylene glycol poisoning cases. These data support the observation that higher populated states had a higher percentage of calls made to the ASPCA poison control center.

Census data provided (USC, 2014) for the state of Wyoming listed Wyoming as the 51st least densely populated state except for Alaska, but having the lowest population with 563,626 people. The ranking of 51 is due to the inclusion of Puerto Rico and District of Columbia in the

population analysis. No calls being received from Wyoming is likely associated with the lack of population mass correlated with call rate.

The American Kennel Club (AKC, 2014) data between 2006 and 2011 were utilized to determine the most popular breeds of dogs during this data set time period. The most popular dog since 1991 was the Labrador retriever, and the remainder of the top five breeds exchanged positions in the list, but all had been top five since 2006. These breeds in no particular order were the Labrador retriever, German shepherd, Golden Retriever, Beagle and Yorkshire terrier. The breed of dog most often seeking treatment for ethylene glycol poisoning in this study was the Labrador retriever at 20.8% followed by the German Shepard at 7.2%. Unspecified dog breeds were at around 8%. Since the Labrador retriever was the most popular breed since 1991 these results were not surprising. In addition, overall, veterinary visits increased for dogs from 2006 to 2011 and decreased for cats (CFAI[a], 2014).

The most popular breeds for cats varied depending upon which data were examined. The Cat Fanciers Association (CFAI[b], 2014) determined the most popular breed according to live births on litter applications, while the Vetstreet data (VSS, 2014) relied on birth rates from over 5,000 clinics across the United States out of the 30,000 possible clinics. First, the data from the Cat Fanciers Association data were examined.

The Cat Fanciers Association data (CFAI[a], 2014) between 2006 and 2011 were utilized to determine the most popular breeds of cats during this data set time period. The most popular cat for the last nineteen years was the Persian, and the remaining of the top five breeds have exchanged positions in the list, but all have been top five since 2006. The top breeds in no particular order were Persian, Exotic, Maincoon, Siamese and Abyssinian. But, the last two years the Ragdoll and Sphinx breeds replaced the Siamese and Abyssinian for positions four and five in popularity. This case study data only yielded the top five breeds of cats as reported by the Cat Fancier's with an exposure rate of 7.8 %. No correlation can be made between the top five cat breeds from Cat Fanciers Data and this case study for predicting exposure of certain cat breeds.

Vetstreet data (VSS, 2014) revealed that the domestic cat was involved in greater than 90% of poisonings which directly correlates with our findings of 90.7% of all cats exposed having a breed designation of domestic for ethylene glycol poisoning. Any breed is susceptible

to poisoning, so the finding reflects that the domestic cat designation of breed overwhelmingly dominates the cat population in homes in the United States.

The three most important parameters when discussing clinical manifestations of ethylene glycol toxicity were dose dependency, amount of ethylene glycol ingested and that the onset of symptoms was almost always sudden. As evident from Tables 2-2 and 2-3, the symptoms were numerous and varied for both the cat and dog. The distinction between clinical signs and complications (kidney failure) which were secondary to the poisoning were unclear in the progression of ethylene glycol poisoning.

In these poison control center data, many similar clinical signs were reported with nearly the same percentage of occurrence for both dogs and cats. Nervous clinical signs for both species were reported about 30% of the time and ataxia and lethargy were 37% and 25% respectively. Digestive clinical signs for dogs and cats were at 23% and 14%, respectively with vomiting reported about 65% of the time for both. During stage one of the poisoning, which was the time period up to about twelve hours, most of the neurological and gastro intestinal clinical signs occurred. Nervous clinical signs reported included: seizures, ataxia, tremors, depressed, lethargy, drunkenness, and abnormal eye movements. The most extensive listing of clinical signs are provided in Veterinary Toxicology Basic and Clinical Principles, Second Edition (Gupta, 2012). This listing has the exact same clinical signs for cats and dogs, except the clinical signs usually seen in stage two for cats and dogs have a different time period. The other clinical signs for stage two and three in cats began at 12-24 hours while for dogs clinical signs they began at 24-36 hours (Gupta, 2012). The list encompassed all the general clinical signs reported in our case study. One clinical sign not reported in poison control center data that was found in the above mentioned book was that kidneys were often swollen and painful, particularly in cats (Gupta, 2012).

During the latter stages, neurological and gastrointestinal clinical signs persisted and cardiopulmonary clinical signs appeared and ran the gamut of possible scenarios. Clinical signs included: tachycardia, bradycardia, hypertension, and hypotension. General clinical signs appearing included dehydration, death, anorexia, hypothermia, hyperthermia and vocalization. By stage two, metabolic acidosis was in full swing and other metabolic conditions had arisen like azotemia, elevated anion gap and hypocalcemia allowing for detection in laboratory testing. In stage three kidney failure was occurring, calcium oxalate crystals were formed, and tubular

necrosis was beginning. Again, the severity of clinical signs was increasing if the animal was still alive.

Two clinical signs listed in this case study of ethylene glycol not readily listed in reviewing the literature of ethylene glycol clinical signs were vocalization and licking. Vocalization was reported as a general clinical sign and general clinical signs make up roughly 14% of the total clinical signs reported. Vocalization makes up 6.5% of general clinical signs. Licking was considered a behavioral clinical sign. Behavioral clinical signs while making up a small number of overall reported clinical signs at 2% revealed a 10% reported clinical sign of licking.

The exposure route findings in this data was similar to that described by the study of Forrester, et al., who recorded that 95% of all poisoning were by way of ingestion and dermal exposure accounted for 5% of the cases (Forrester, 2004). Ingestion was the primary route of exposure listed in many human studies as well. The annual reports of the American Association of Poison Control Centers (AAPCC) from 2004 to 2009 reported that ingestion was the route of exposure in (76% - 84 %) of human cases, followed by dermal (7% - 8%) (Bronstein, 2010, 2009, 2008, and 2007), (Lai, 2006) and (Watson, 2005).

In the examination of data provided by the ASPCA for ethylene glycol possible ingestion of domestic animals from 2006-2010, dermal exposure accounted for roughly 4% of the total exposure routes. Absorption studies in mice and humans revealed that the absorption through skin was not enough to cause poisoning. The rate was so slow that the body was able to eliminate the ethylene glycol, metabolites did not accumulate and poisoning did not occur. The dilution of ethylene glycol by 50% as it found in antifreeze reduced the absorption by half (Sun, 1995).

Conclusion

This study supported previous findings that cats and dogs were the most commonly exposed animals regardless of type of poisoning (Leth, 2005). Oral and dermal were the most common routes of exposure for general poisonings and specifically ethylene glycol poisoning. Exposures throughout the year were fairly consistent with previous findings, although ethylene glycol poisoning seemed to be consistent all year long without a peak in the summer, winter or

the Christmas holiday. The higher populated states made more calls to the poison center than lower populated states.

In researching ethylene glycol cases, few comprehensive listings or compiled data of the most often clinical signs encountered in ethylene glycol poisoning were available for either cats or dogs. But, a few data sets did allow for an attempted ranking of the clinical signs. It was not possible to correlate the most common breeds of cats and dogs and make determinations about the prevalence of ethylene glycol poisonings from these data. Total ethylene glycol exposures over this six year time period would indicate that only 0.00096% of the cat and dog total population would be exposed to ethylene glycol over a six year time period. Dogs would account for 0.00084% and cats would account for 0.00012% of exposures.

Tables

Table 2-1: Numbers and percentages of species involved in Ethylene Glycol poisoning for 2006 to 2011.

Species	No	%
Dog	1522	87.07%
Cat	216	12.36%
Ferret	2	0.11%
Horse	2	0.11%
Pig	1	0.06%
Raccoon	3	0.24%
Unknown	2	0.11%
Total	**1748**	

Table 2-2: Clinical signs breakdown for dogs suspected of Ethylene glycol poisoning from 2006 to 2011.

Clinical Sign	2006	2007	2008	2009	2010	2011	Total
Bhavor	6	4	4	7	7	1	29
Cardio	9	4	5	3	5	7	33
Digest	70	50	48	53	50	40	311
DND	3	1	2	1	2	1	10
Gneral	51	23	23	23	35	21	176
Hemato	6	3	5	6	12	8	40
Metab	34	20	12	12	30	18	126
MusSkl	11	4	3	3	4	7	32
Nerve	98	54	60	70	83	54	419
Repro	0	0	1	0	0	0	1
Respir	9	0	4	2	5	6	26
Senses	3	1	2	0	3	3	12
Skin	2	0	0	0	2	2	6
Urinry	33	26	17	20	38	27	161
(blank)	210	154	180	173	175	172	1064
Total	**545**	**344**	**366**	**373**	**451**	**367**	**2446**

Table 2-3: Clinical signs breakdown for cats suspected of Ethylene glycol poisoning from 2006 to 2011.

Clinical Sign	2006	2007	2008	2009	2010	2011	Total
Bhavor	2	0	1	1	4	1	9
Cardio	3	1	0	0	2	0	6
Digest	16	5	8	15	7	5	56
Gneral	21	4	10	12	15	3	65
Hemato	0	0	5	0	2	1	8
Metab	11	3	7	8	14	6	49
MusSkl	4	3	0	1	0	1	9
Nerve	34	14	17	30	29	7	131
Respir	0	2	2	2	2	3	11
Senses	3	3	1	1	2	1	11
Skin	0	0	2	0	0	1	3
Urinry	14	3	4	6	5	3	35
(blank)	26	8	23	11	19	11	98
Total	**134**	**46**	**80**	**87**	**101**	**43**	**491**

Figures

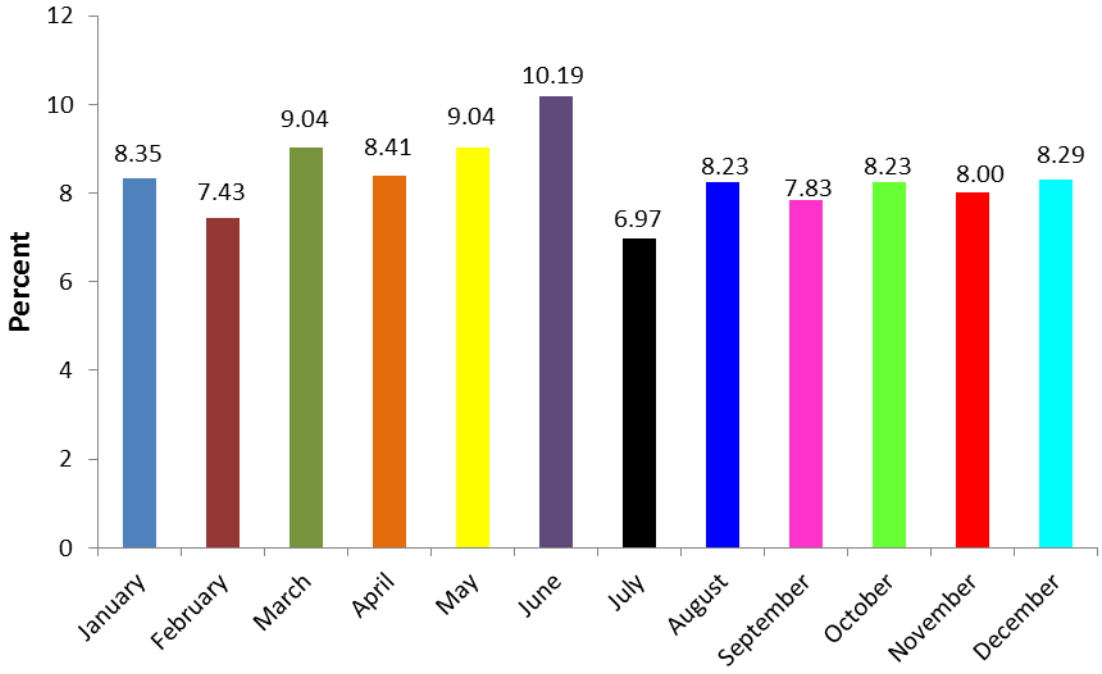

Figure 2-1: Percentages of 1737 calls by month for the United States and Canada between 2006 and 2011.

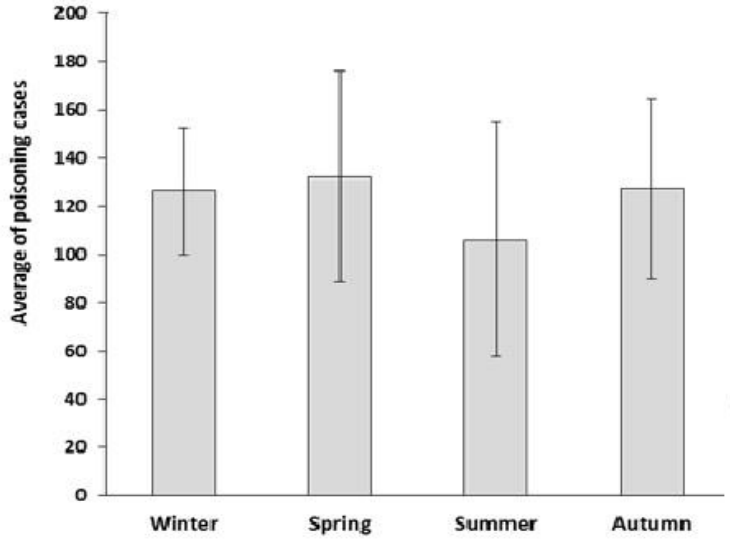

Figure 2-2: Average Seasonal Ethylene Glycol Poisonings in Dogs and Cats for the years 2006 – 2011. Not statistically significant data.

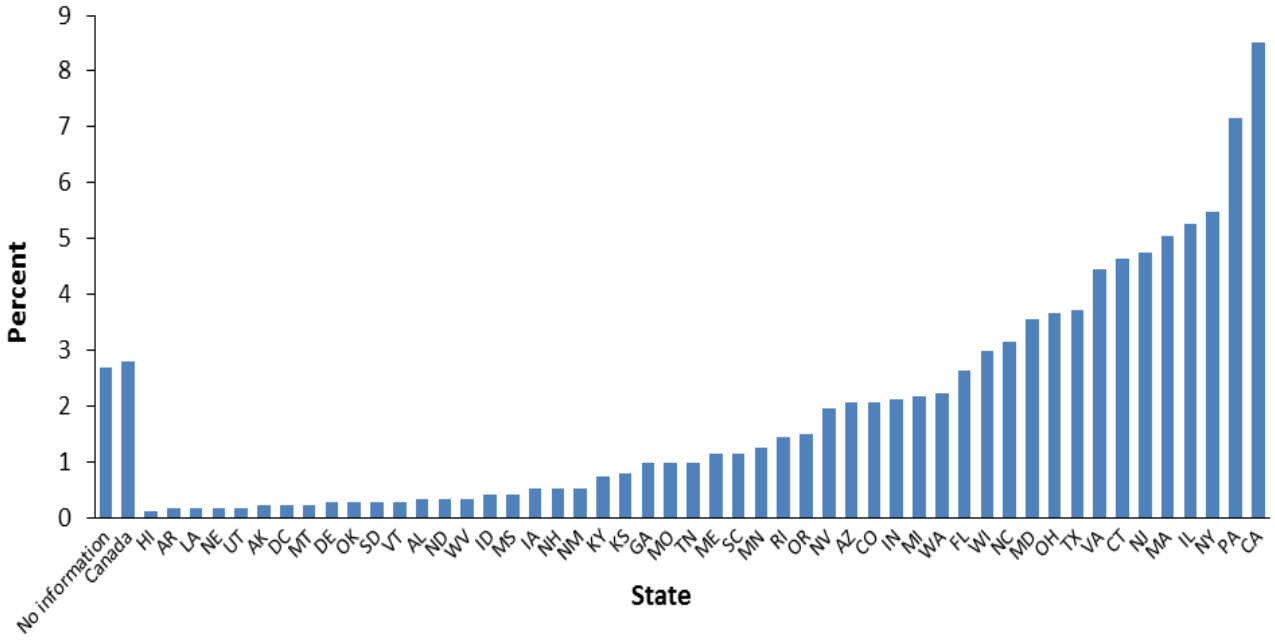

Figure 2-3: Percentage of calls received by state in the United States and Canada between 2006 and 2011.

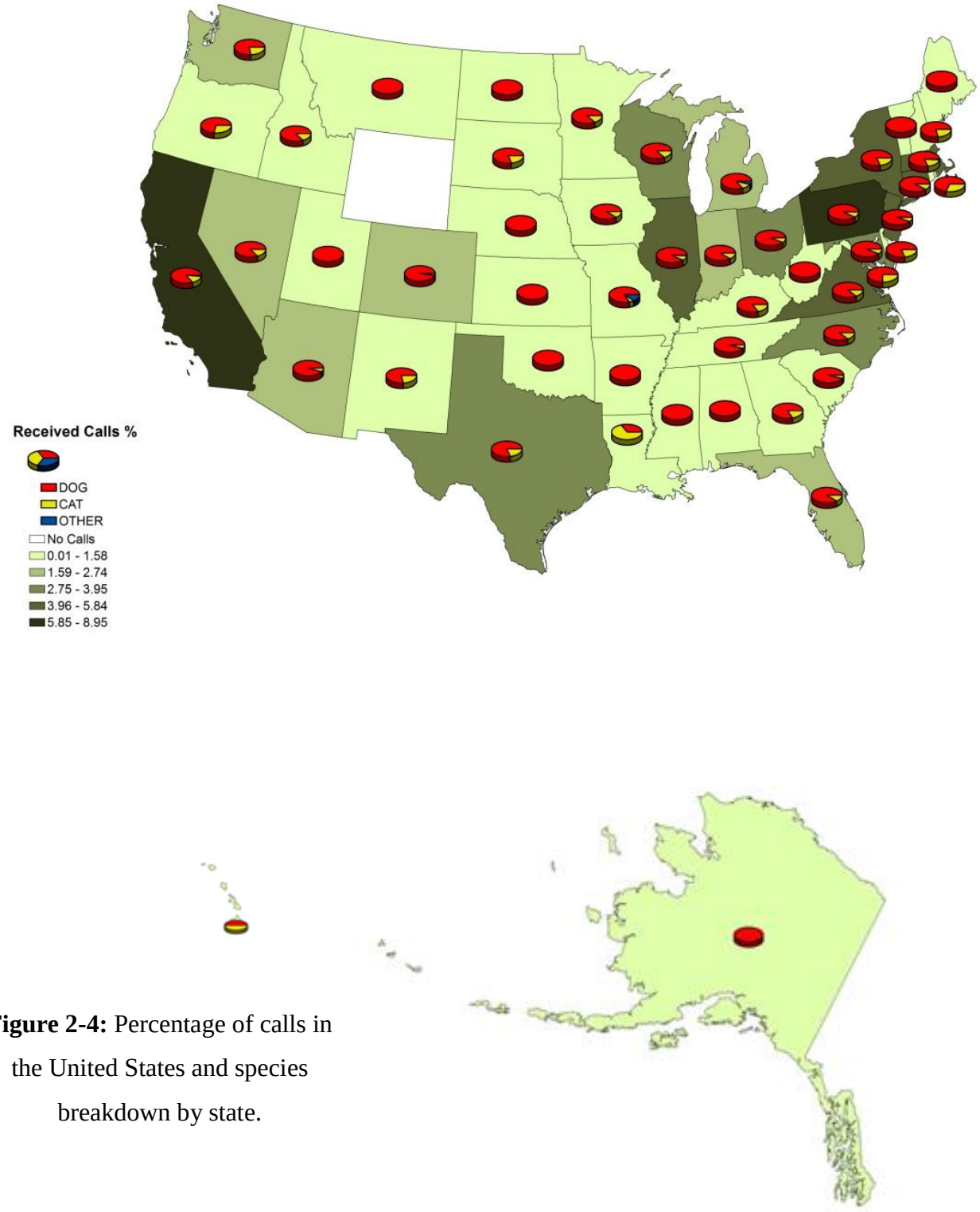

Figure 2-4: Percentage of calls in the United States and species breakdown by state.

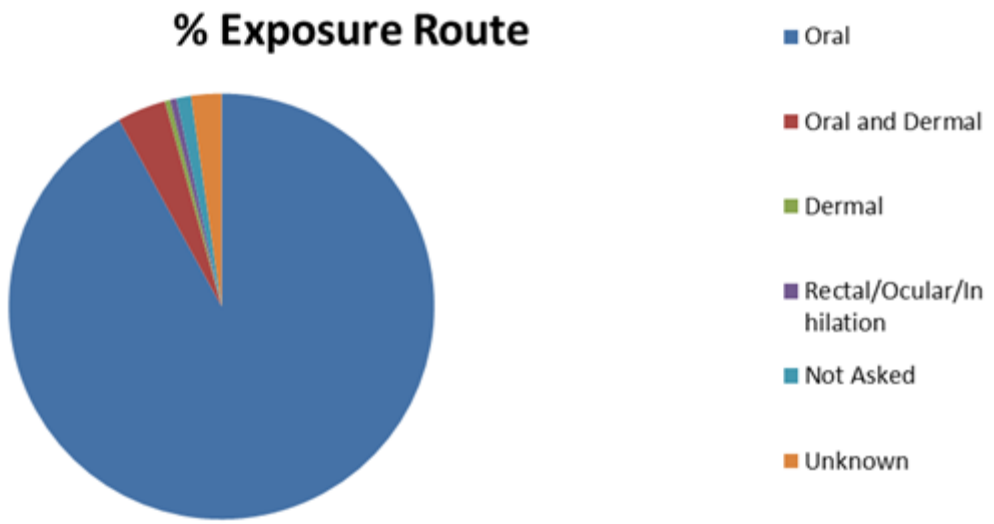

Figure 2-5: Exposure routes for cats and dogs suspected of Ethylene glycol poisoning from 2006 to 2011.

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Chapter 3 - Rapid Methods of Detection of Ethylene Glycol Poisoning

This chapter outlines an Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy method for calcium oxalate crystal identification in renal tissue and urine that was simple, rapid and highly adaptable. The method allowed for the differentiation between calcium oxalate crystals and other crystal types that may be found in the urinary system. Further advantages included little to no sample preparation, no calibration of the instrument and increased sensitivity. This method can be applied to urine, fresh renal tissue, and renal tissue samples that have been fixed in 10% buffered formalin and mounted in paraffin. This chapter also outlines an ATR-FTIR spectroscopy method for identifying non-metabolized ethylene glycol present in vomitus and stomach contents.

Introduction

Ethylene glycol products are commonly found in households, primarily as the main component of automotive antifreeze. Other sources include de-icing solutions, solvents for paints and stains, hydraulic fluid, and many others. Animals readily consume products that contain ethylene glycol due to its sweet pleasing taste. Following ingestion ethylene glycol is rapidly absorbed from the gastrointestinal tract. A fraction of absorbed ethylene glycol is excreted unchanged through the urine (Grauer, 1987), while the remainder is metabolized. The book, Small Animal Toxicology, Third Edition (Peterson, 2013) reports that as much as 50% of the ethylene glycol can be excreted unchanged. Ethylene glycol is metabolized into glycol-aldehydes and organic acids, such as oxalic acid, by alcohol dehydrogenase and other enzymes in the liver. Oxalic acid reacts with calcium to form insoluble ca-oxalate crystals that are deposited in tissues, particularly renal tissue, and excreted into urine (Davis, 1997).

Ca-oxalate deposits remain in tissues when ethylene glycol and other metabolites of ethylene glycol have been eliminated (Davis, 1997). Deposits in renal tissue are typically observed histologically as crystals in the tubules. The presence of large crystal deposits usually at least 3% is an important post mortem diagnostic feature in poisoned animals, along with signs of renal tubular degeneration and necrosis. In addition to ca-oxalate deposition in tissues, ca-

oxalate crystalluria is a common feature in poisoned animals, *ante mortem* as well as *post mortem*, and can provide valuable diagnostic evidence. Substances other than ca-oxalate may, however, form crystals in the urinary system. Among these are struvite, cystine, ammonium urate, xanthine, and silicium dioxide (Hesse, 1990).

Struvite, oxalate, and urate crystals are often found in the urine of cats and dogs. Struvite crystals are composed of magnesium, ammonium and phosphate and can be controlled with diet. These crystals are found in alkaline urine and may join together to form stones. A prescription diet low in magnesium will help to decrease the pH of the urine and resolve the struvite crystal formation, but as the urine becomes more acidic the formation of oxalate and urate crystals increases. High levels of calcium, protein, sodium and vitamin D along with acidic urine favor the formation of oxalate crystals (Osborne[a], 2009). Urate crystals are more commonly found in dogs and usually associated with liver disorders and metabolic diseases.

Xanthine crystals are a natural by-product of purine metabolism which is normally converted to uric acid (Osborne[b], 2009). These crystals also form stones and prefer an acidic environment. Diets high in protein have an increased risk for xanthine crystal and stone development. Cystine is also normally found in the body and forms crystals that may also combine and form stones. Drinking large amounts of water can help to prevent stone formations for all types of crystals, but it is difficult to coerce an animal to drink more water.

Ca-oxalate has a unique infrared absorption spectrum compared to other crystals, and infrared spectroscopy has been used to characterize identify ca-oxalate in urine (Volmer, 2001). This section outlines an ATR-FTIR method for the detection and confirmation of ca-oxalate crystals in both kidney tissue and urine. Un-metabolized ethylene glycol testing was also attempted in a vomitus sample, but it was not found to be a successful method.

Methods

Kidney tissue and urine from animals that died of renal failure, following exposure to ethylene glycol, were obtained from cases submitted to the Kansas State Veterinary Diagnostic Laboratory. Kidney tissues were examined as fresh, and also were also examined as dried at 95^0C for 4 hours in a convection oven, and separate tissues were examined as 10 micron sections mounted on glass slides following routine fixation in 10% buffered formalin and mounted in

paraffin wax. Urine was centrifuged at 3,000 *g* for 10 minutes in an Eppendorf model 5415R, the precipitate was collected for analysis from the bottom of the centrifuge tube and dried on a watch glass for two hours in a 60°C Fisher Scientific Isotemp convection oven. Sample spectra were compared to a pure ca-oxalate reference (Figure 3.2). The standard was a calcium oxalate monohydrate powder from Fisher Certified Reagents with catalog number C-112.

Fresh kidney samples submitted were thinly cut with a razor blade and placed in contact with the instrument and then examined against known calcium oxalate peaks. The same fresh samples were also analyzed microscopically on an Olympus SZX16 stereo microscope to locate calcium oxalate crystals present in the kidney tissue.

ATR-FTIR spectra were obtained using a Nicolet 6700 FTIR spectrometer (Thermo-Fisher), fitted with a diamond crystal GladiATR ATR module (Pike Technologies). Following background spectra collection, samples were placed on the diamond surface of the ATR module, and clamped onto the crystal. Absorbance spectra were collected between 4,000 and 450 cm^{-1} at a spectral resolution of 4 cm^{-1}. Thirty-two scans were co-added and averaged. Infrared absorption between 1330 and 1290 cm^{-1} was used for ca-oxalate identification in samples.

To evaluate the ATR-FTIR method for its usefulness, the specificity, sensitivity, accuracy, misclassification and predictive values were calculated from examination of twenty formalin-fixed and paraffin-wax mounted kidney samples (10 positive and 10 negative, as determined by a single pathologist using traditional light microscopy), were analyzed by a blinded ATR-FTIR operator and grouped into positive or negative samples based on the ca-oxalate identification peak, and using three times background as the criterion for defining a sample as positive.

Testing for un-metabolized ethylene glycol in a vomitus sample was attempted. The sample was run and compared to an aliquot of ethylene glycol using a serial dilution of Fisher Certified Ethylene Glycol with catalog number E-178. The method used was identical to the method used for ca-oxalate identification, except a 20ul aliquot was placed on the diamond surface of the ATR-FTIR module and no sample preparation other than filtering using Whatman #1 filter paper or a Nalgene rapid-flow disposable filter units was required. The wavenumbers used in the detection of un-metabolized ethylene glycol in vomitus were 1084 cm^{-1}, 1039 cm^{-1}, and 882 cm^{-1}.

The validity of ATR-FTIR method for the detection and confirmation of ca-oxalate crystals was evaluated by measuring the specificity (Sp), sensitivity (Sn), negative predictive values (PV-), positive predictive values (PV+), accuracy (Ac), and misclassification (Misc). The values were calculated according to the Table 3-1 and following equations:

Table 3-1: Classification of individual animals according to test results and disease status

	Disease status		
Test Result	**Diseased (D+)**	**Non-Diseased (D-)**	**Total**
Positive	True Positive (TrP) **8**	False Positive (FP) **0**	Total Positive (TP) **8**
Negative	False Negative (FN) **2**	True Negative (TrN) **10**	Total Positive (TN) **12**
Total	Total Diseased (TD+) **10**	Total Non-Diseased (TD-) **10**	Grand Total (GT) **20**

The equations:

Sp = TrN / TD-

Se = TrP / TD+

PV+ = TrP / TP

PV- = TrN / TN

Ac = (TrP + TrN) /GT

Misc = (FP + FN) /GT

Results

Figure 3.1 demonstrated the calcium oxalate peak used for diagnostic determination of ethylene glycol poisoning. Ca-oxalate formed a unique infrared absorption peak, compared to kidney tissues and tissue-mounting matrix between 1330 and 1290 cm^{-1} (Figure 3.4), and more specifically around 1313 cm^{-1}.

The ATR-FTIR specificity for the twenty paraffin kidney samples was 100%, and sensitivity was 80% compared to traditional light microscopy performed by a licensed pathologist for ca-oxalate crystal identification (Table 3.1 and 3.2). The specificity referred to the ability to detect those animals not diseased, while sensitivity referred to the ability to detect

those animals that are diseased. The accuracy of the test was 90%, with misclassification at 10%. The accuracy referred to the proportion of animals correctly identified by the test, and the misclassification referred to the proportion of animals not correctly identified by the test. The predictive values (PV+, PV-) were 100% (confidence interval 67.5% - 100%) and 83% (confidence interval 55.2% - 95.3%) respectively. PV+ indicated what proportion of positive animals were diseased, and PV- indicated what proportion of the tested negative animals were correct.

The calcium oxalate detection in kidney samples was a novel application performed at Kansas State University. The usefulness of the test was compared to the traditional light microscopy performed by a pathologist and found to be a comparable procedure. The confidence intervals for these data were performed on a limited study on an initial application. Further testing would have to be done to confirm the confidence intervals as a true representation of these data.

Since June 2011, when the methods in the appendix were written, only nine total samples were submitted for ethylene glycol poisoning testing of which eight were kidneys and one was urine. Of the nine submissions, eight were canine and one was a feline. Seven kidneys from deceased dogs were submitted and five were positive. Crystals were detected microscopically and confirmed by the ATR-FTIR. A urine sample was submitted from a dog, but it was tested and found to be negative according to the procedure listed in Appendix A. One kidney sample from a cat was submitted and found to have calcium oxalate crystals that were detected both microscopically and on the FTIR. All positive samples were identified microscopically and confirmed by the FTIR.

Only one known positive sample was submitted for ethylene glycol testing along with an un-metabolized sample. A vomitus sample was submitted along with a kidney from a dog. The kidney sample tested positive for ethylene glycol poisoning, but ethylene glycol was not detected in the vomitus sample of the same animal. The detection limit for ethylene glycol on the FTIR was 9990 ppm. On the positive control spectra there was a small ill-defined peak at 5000 ppm and a well-defined peak at 9990 ppm. Due to the low submission numbers and a lack of detection, further data are needed to predict its reliability as a proper testing method. Peaks at 5000 ppm and 9990 ppm were shown along with the vomitus sample. Concentrations in the samples at the time of testing were too low to be detected as ethylene glycol on the ATR-FTIR

(Figure 3.5). However, the kidney sample from the same dog was confirmed positive on the ATR-FTIR using test method TM003.03.

Discussion

ATR-FTIR spectroscopy for ca-oxalate crystal identification in tissues and fluids is a highly specific method that can be adapted to a variety of tissue processing methods (Figure 3.1). The ability to confirm the identity of crystals on cut surfaces of fresh kidney tissue and urine offers the advantage of rapid identification requiring minimal sample processing.

Due to the many crystals that can be found especially in urine, this method can be extremely valuable for the timely diagnosis and treatment of poisoned animals. The ease of instrument operation gives reliable, rapid and reproducible results. The already present internal calibration and lack of sample preparation makes this a relatively quick and easy test.

Microscopic crystal identification requires operator experience and even then may not be sufficient for positive identification of crystals, as the identification is somewhat subjective due to the many and varied morphologies crystals can yield. ATR-FTIR, in comparison, requires minimal operator experience and training, and the procedure is relatively rapid. There is also less subjectivity in the interpretation of results, which reduces inter-operator variability.

ATR-FTIR may not detect ca-oxalate crystals if the crystal density is low. The fact that crystals may not form at all or may form in low concentrations is an obvious limitation to the methods created in this project. However, crystal density is usually sufficient for detection in cases of ethylene glycol poisoning, which is the most common cause of excessive ca-oxalate accumulation in kidney tissue. Sensitivity can be increased by drying kidney tissue as is shown in Figure 3.3 thus increasing the likelihood for detection. In suspect cases of poisoning where crystals are not present, the diagnosis should be left up to histological examination. Combination of symptoms, histological examination and testing should be used to make a definitive diagnosis.

In a comparison of paraffin fixed samples determined by light microscopy to be positive and negative, and the determination of the same slides by the ATR-FTIR in the identification of ethylene glycol poisoning, two of the positive slides were misdiagnosed on the FTIR. Possible reasons for this could include misidentification by the pathologist initially when identifying a negative as a positive. Calcium oxalate crystals may have been present in the kidney tissue, but

the sample was tested on the ATR-FTIR in a location on the sample where no crystals were present. The camera on this particular instrument did not provide a clear picture of the crystals in the tissue samples, which made it difficult to determine the correct location to test. This issue limited the effectiveness of the operator's ability to perform testing.

Attaching a microscope directly to the instrument would aid in locating crystals on tissue, and thus enabling a more accurate test. Also, testing the samples in various locations on the tissue could prevent a misidentification. Any further testing on paraffin encased kidney ethylene glycol suspected samples needs to include a protocol for multiple testing sites.

With only one vomitus sample tested, the ATR-FTIR did not seem to be a viable instrument for detecting ethylene glycol in a vomitus sample. The detection limit of the instrument could be much higher than what was present in the sample, or perhaps there was none in the sample at the time of testing due to already being metabolized. Detection of a peak was not distinguishable until 5000 ppm with well-defined peaks at 9990 ppm. The inability to detect ethylene glycol in vomitus may be due to the fact that the vomiting occurred after the metabolism of ethylene glycol was nearly completed. When vomiting is due to a non-gastric problem such as poisoning, vomiting occurs after the brain signals a need to do so and not as an automatic response. Kidney failure can also spur on vomiting, but kidney failure due to ethylene glycol poisoning would occur late in the clinical signs after absorption of ethylene glycol would have already occurred.

Another limitation of ATR-FTIR testing includes a higher signal to noise ratio spread out over the spectrum due to the rapid and broad scanning of wavelengths, but this prevents a large S/N ratio from overshadowing peaks in a localized area of the spectrum. Detected peaks may be due to noise or infrared radiation detection from other sources and background. For diagnostic purposes urine and kidney detection of ca-oxalate are considered much more reliable samples than detection of ethylene glycol in vomitus or stomach contents for a poisoning diagnosis or confirmation of poisoning. Both the urine and kidney samples were accurately able to detect calcium oxalate crystals, leading to a stable and reliable method for determining ethylene glycol poisoning.

Table 3-2: Raw data of ATR-FTIR method for Ca-oxalate crystal identification in fixed, wax-mounted samples, compared to traditional microscopy

Results	No.
True positive	8
False positive	0
True negative	10
False negative	2
Total	20

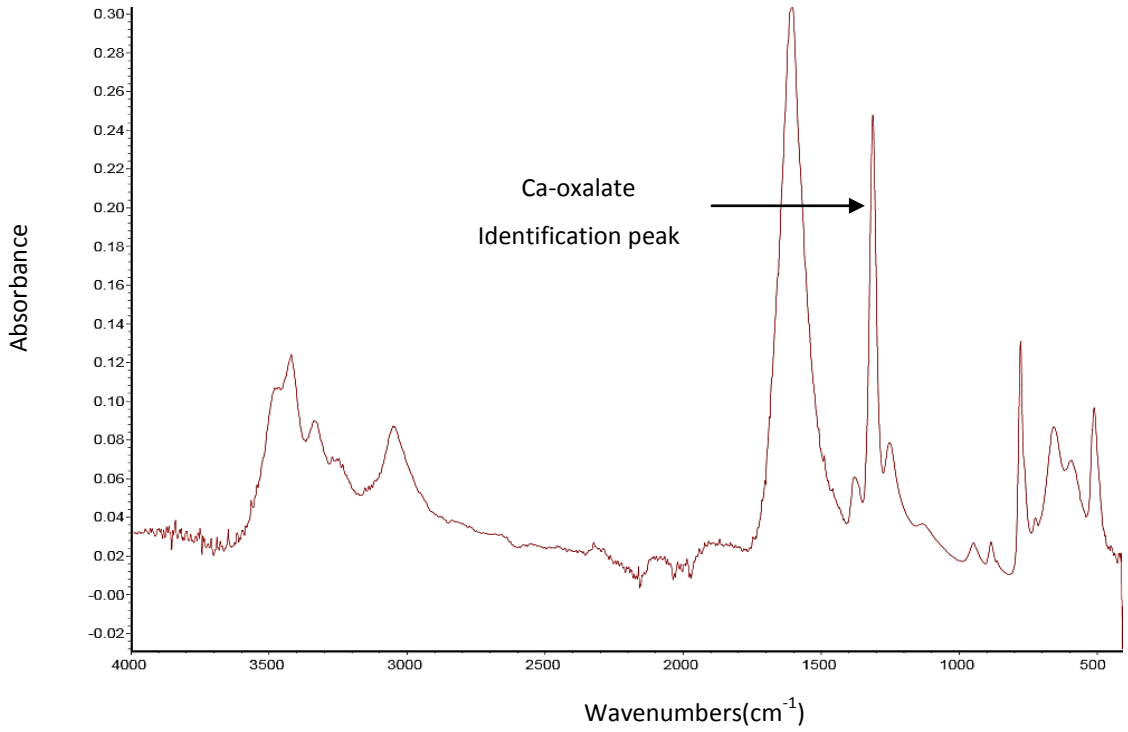

Figure 3-1: ATR-FTIR spectra demonstrating a pure Ca-oxalate reference and the peak used for Ca-oxalate identification in tissues and urine between 1330 and 1290 cm^{-1}

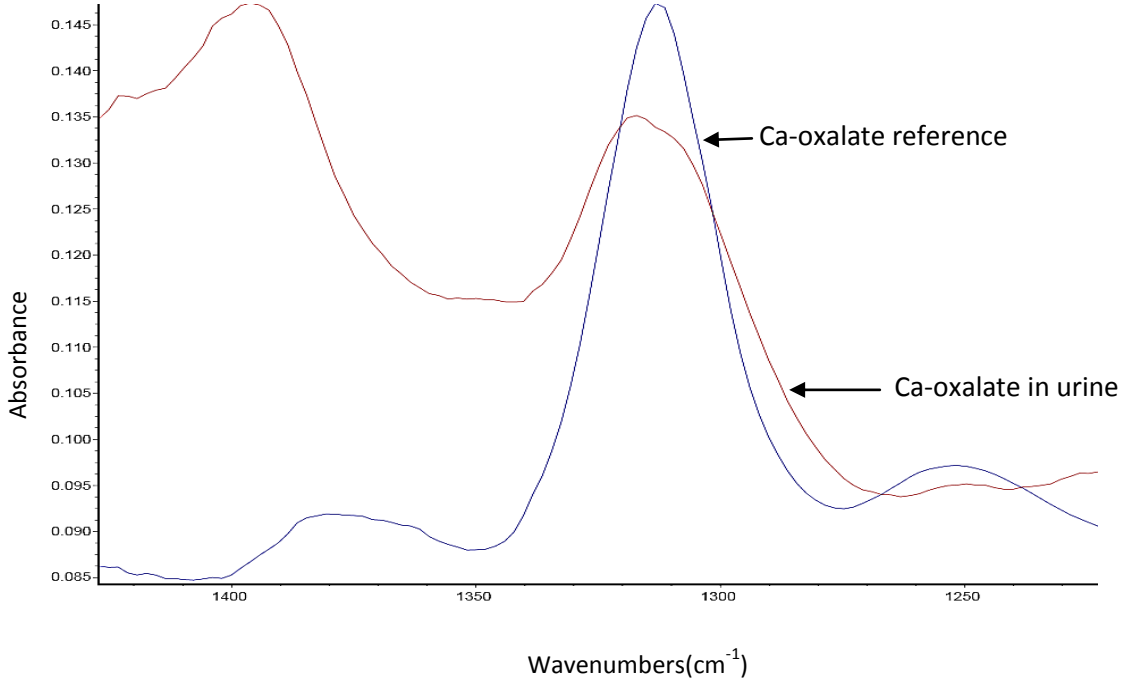

Figure 3-2: ATR-FTIR spectra demonstrating Ca-oxalate in horse urine

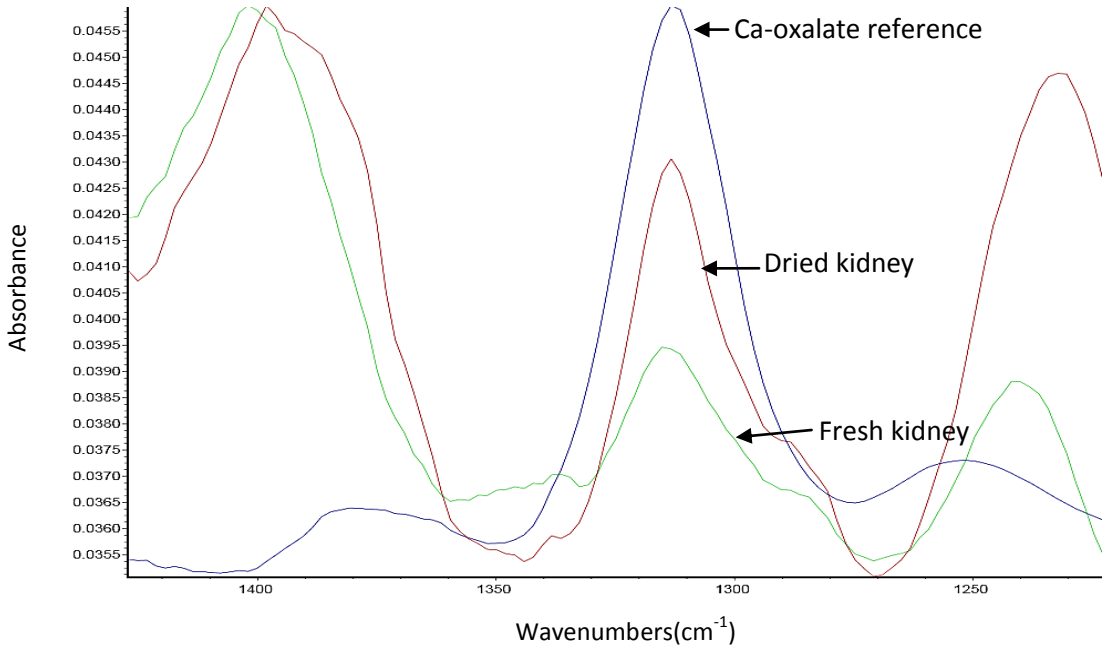

Figure 3-3: ATR-FTIR spectra demonstrating fresh kidney and dried kidney from a cat that died of ethylene glycol poisoning

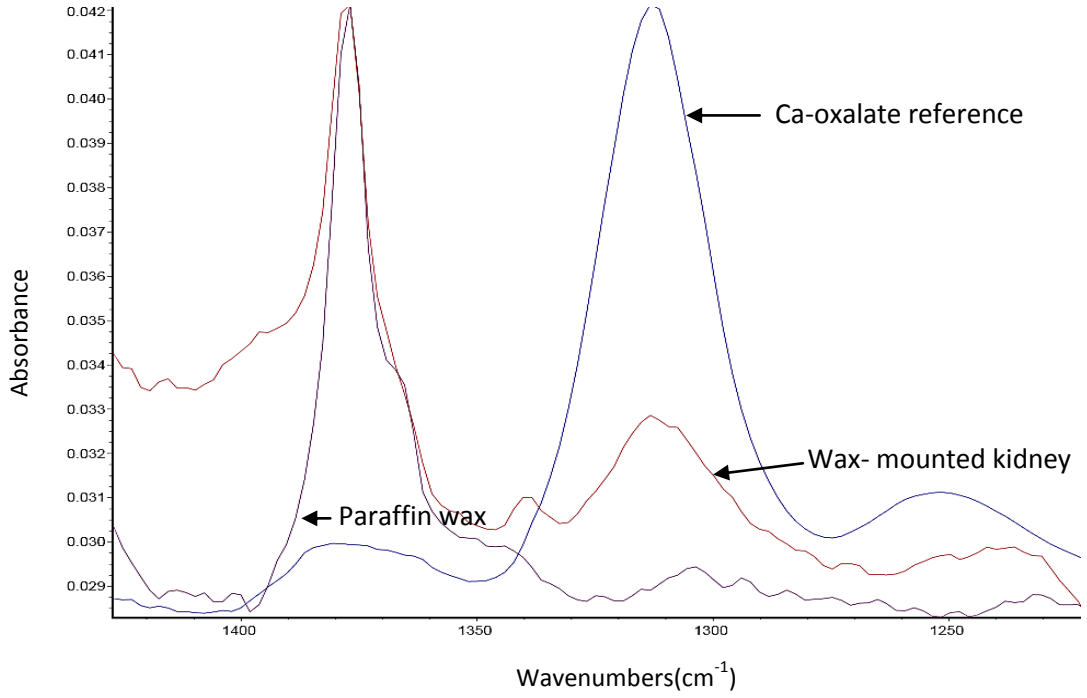

Figure 3-4: ATR-FTIR spectra demonstrating paraffin wax and wax-mounted kidney from a dog that died of ethylene glycol poisoning

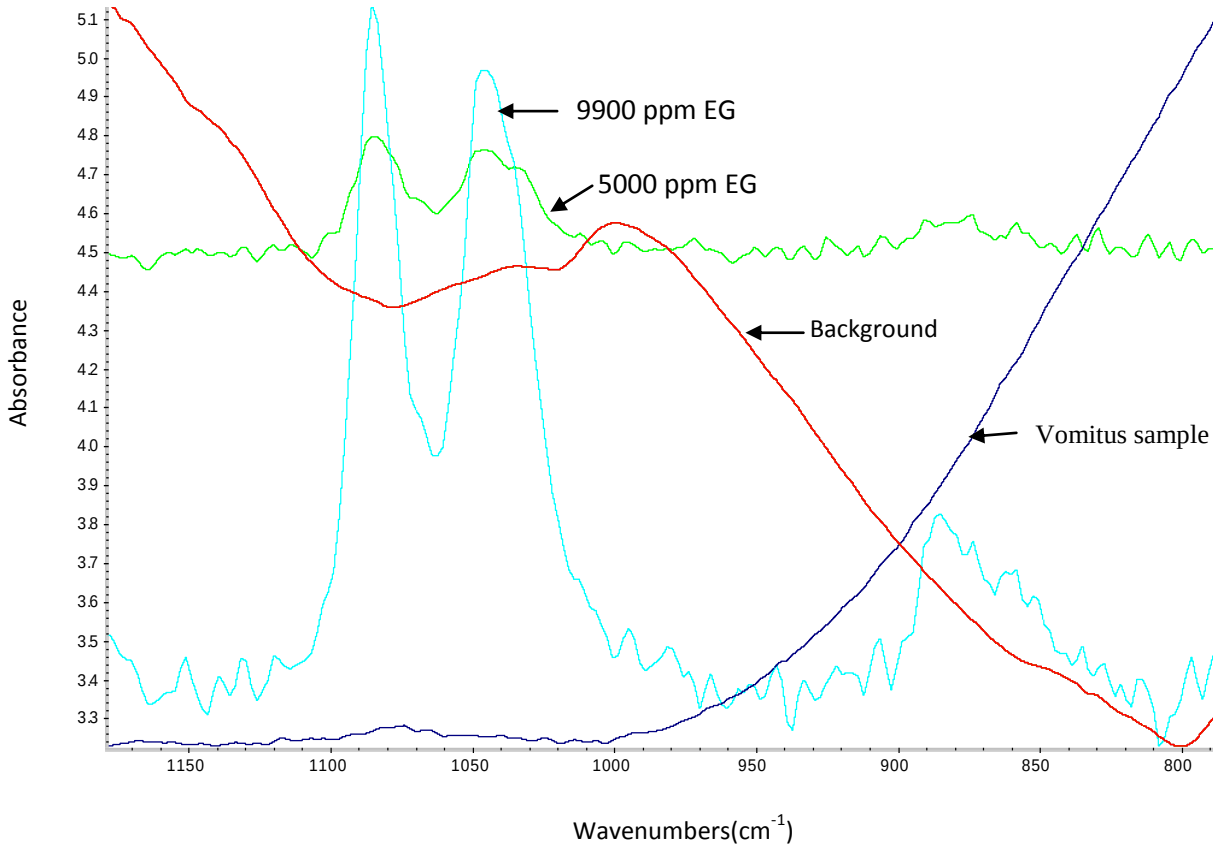

Figure 3-5: Ethylene glycol detection at 2500 ppm, 5000 ppm and vomitus sample of a dog that died from Ethylene Glycol Poisoning

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Appendix A - Determination of calcium oxalate in kidney tissue and urine using ATR-FTIR

DATE: July 25, 2013

TITLE: Determination of calcium oxalate in kidney tissue and urine using ATR-FTIR

TEST METHOD: TM 003.3

AUTHOR/
CONTACT: Lori Blevins/532-5678

I. PURPOSE

This method is to be used for determination of calcium oxalate in submitted diagnostic samples, including urine and kidney. Calcium oxalate crystals in kidney tissue and urine is often associated with ethylene glycol and oxalate poisoning.

II. DEFINITIONS

- **ATR-FTIR**: (Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy) The mid-infrared, approximately 4000–400 cm^{-1} (30–2.5 μm) exploits the fact that molecules absorb specific frequencies that are characteristic of their structure.
- **EG**: Ethylene glycol
- **DDI**: Double distilled water
- **ppm**: Parts per million
- **Birefringence**: decomposition of a ray of light into two rays when it passes through certain types of material.

III. HAZARDS AND SAFETY PRECAUTIONS

Be sure to wear safety glasses, lab coat, and gloves when handling reagents.
Read all MSDS's as many of these reagents have inhalation exposure hazards.

IV. PROCEDURE

A. Urine Testing

1. Equipment/Supplies
   a. Centrifuge
   b. Centrifuge Tubes
   c. IsoTemp Drying Oven
   d. ATR-FTIR
   e. Compound Microscope
   f. Stereo microscope
   g. Watch glasses
   h. Spoonula or equivalent
   i. DDI water
   j. Polarizing lens

2. Sample Preparation
   a. Centrifuge urine sample(s) until crystals collect at the bottom of the tube.
   b. Use spoonula to remove the crystals and place them on a watch glass. DDI water can be used sparingly to rinse urine from crystals.
   c. Dry the crystals for two hours at 60˚C.
   d. Remove from oven and let cool.

3. Data Collection
   a. Prepare to run on ATR-FTIR and refer to Use, Maintenance, Performance Verification and Calibration of the FT-IR (LP 2075kk) for guidance.
   b. Load calcium oxalate crystal from database to use as reference.
   c. Collect a background spectrum.
   d. Place sample crystals on ATR diamond surface, clamp and verify crystal contact using the video monitor.
   e. Collect a sample spectrum.
   f. Examine crystals under the compound microscope and stereo microscope and compare to known calcium oxalate crystals for identification.
   g. Should see birefringence when looking through polarized light.

B. Kidney Testing

1. Equipment/Supplies
   a. ATR-FTIR
   b. Stereo Microscope
   c. Scissors/Razor Blade

2. Sample Preparation
   a. Slice or cut a piece of tissue thinly.
   b. If crystals are visible using stereo microscope then perform testing in that location.

c. Determination can also be made on fixed tissues.

3. Data Collection
   a. Load calcium oxalate crystal from database to use as reference.
   b. Collect a background spectrum.
   c. Place sample on ATR diamond surface, clamp and verify crystal contact using the video monitor.
   d. Collect a sample spectrum.

V. DATA COMPILATION

1. Compare spectra of samples to known spectra of calcium oxalate to determine if peaks are consistent with submitted samples.
2. Examine microscopically for further determination.
3. A printed report for each analysis should be kept upon completion of analysis.
4. Keep copies and attach to submission forms.
5. Copies can be released to client or a spreadsheet form can be released if it makes interpretation easier. Otherwise, data will be entered into a data management system as a result or at least as a comment.
6. Send reports to Toxicologist for interpretation, as needed.

VI. QUALITY CONTROL

- The bands primarily used in the detection of calcium oxalate are 1313 cm^{-1} and 778 cm^{-1}.

- Peaks should be at least 3 times above background to be significant. Interferences at bands of interest require the use an alternative band, if available. If both bands are masked by interferences the method should not be used.

- Sample suitability for testing and rejection will be determined on an individual basis.

- Retests will be performed if the toxicologist deems it necessary and as sample size permits.

- Testing has been evaluated in-house for dog tissue and urine and also for horse urine.

VII REFERENCES

Use, Maintenance, Performance Verification and Calibration of the FT-IR (LP 2075kk).

VIII. FORMS AND REPORTS-NA

IX. DISTRIBUTION OF CONTROLLED COPIES

A. Originals of signed current versions of SOPs will be kept in the office of the Quality Manager

B. Copies of signed current versions of SOPs will be:
Distributed to appropriate personnel as determined by the author, Laboratory Supervisor and the Quality Manager

X. ARCHIVING

A. The Laboratory Supervisor and Quality Manager should be notified of all SOPs that are eligible for retirement or revision

B. Retired SOPs and superceded versions of SOPs will be maintained as described in SOP 103 Document control.

XI. SUMMARY OF REVISIONS

Date	Description of Change (include page/section information)
05/19/2011	Retitled.
07/25/2013	Took out UVIS and added Data Management System.

Approved by: _____ Date: _____

Appendix B - Determination of ethylene glycol using ATR-FTIR

DATE: May 19, 2011

TITLE: Determination of ethylene glycol using ATR-FTIR

TEST METHOD: TM 002.2

AUTHOR/
CONTACT: Lori Blevins/532-5678

I. PURPOSE

This method is to be used for determination of ethylene glycol (EG) in submitted diagnostic samples, including environmental samples, vomitus, and stomach contents. EG is commonly found in anti-freeze products. A dedicated kit exists for determination of EG in plasma. This method will not be used to replace plasma testing, but will be used to support a diagnosis when other samples are sent along with blood/serum/plasma samples, or when samples are submitted to substitute for a diagnosis when no blood/serum/plasma is available. ATR-FTIR detection of EG will provide a means of supporting a diagnosis EG poisoning.

II. DEFINITIONS

- **ATR-FTIR**: (Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy) The mid-infrared, approximately 4000–400 cm^{-1} (30–2.5 μm) exploits the fact that molecules absorb specific frequencies that are characteristic of their structure.
- **EG**: Ethylene glycol
- **DDI**: Double distilled water
- **ppm**: Parts per million
- **C-O Functional Group:** Carbon/Oxygen. There are numerous patterns of atoms and bonds that are found frequently in organic compounds. These configurations of atoms are called functional groups, as each has specific, predictable properties.

III. HAZARDS AND SAFETY PRECAUTIONS

Be sure to wear safety glasses, lab coat, and gloves when handling reagents.
Read all MSDS's as many of these reagents have inhalation exposure hazards.

IV. PROCEDURE

C. Vomitus/Stomach Content Testing

1. Equipment/Supplies
    a. ATR-FTIR
    b. Glass Funnel
    c. Whatman #1 filter paper
    d. 20uL pipette
    e. 2-100 uL tips
    f. Beaker or equivalent
    g. Nalgene filtration product
    h. EG positive reference sample

2. Sample Preparation
    a. If needed to obtain a liquid sample, set up funnel apparatus with filter paper, and filter stomach contents or vomitus.
    b. A nalgene filtration product can be used when liquid volumes are low.

3. Data Collection
    a) Prepare to run on ATR-FTIR and refer to Use, Maintenance, Performance Verification and Calibration of the FTIR (LP 2075kk) for guidance.
    b) Collect a background spectrum
    c) Place a 20µl drop of liquid on the ATR crystal surface
    d) Collect a sample spectrum
    e) Place a 20µl drop of EG on the ATR crystal surface
    f) Collect a positive control spectrum

V. DATA COMPILATION

7. Load EG spectra from database to use as reference.
8. Compare spectra of samples and positive control to known spectra of EG to determine if peaks are consistent with submitted samples.
9. A printed report for each analysis should be kept upon completion of analysis.
10. Keep copies and attach to submission forms.
11. Copies can be released to client or a spreadsheet form can be released if it makes interpretation easier. Otherwise, data will be entered into UVIS as a result or at least as a comment.
12. Reports may be sent on to Toxicologist for interpretation as needed.

VI. Quality Control

- EG detection using plasma is considered positive for poisoning at 20 mg/dl (200 ppm) for cats and 50 mg/dl (500 ppm) for dogs using the test strip tests provided by KACEY Anti-Freeze Test Strips. The detection limit on the FTIR for EG in serum is about 10 times that value. So, the FTIR is not suitable for confirming EG poisoning by analyzing blood/serum or plasma.

- The bands primarily used in the detection of EG are 1084 cm^{-1}, 1039 cm^{-1}, and 882 cm^{-1}, and can be used when testing samples in which EG have not been metabolized.

- Peaks should be at least 3 times above background to be significant. Interferences at bands of interest require the use of alternative bands, if available. If all bands are masked by interferences the method should not be used.

- Sample suitability for testing and rejection will be determined on an individual basis.

- Retests will be performed if the toxicologist deems it necessary and as sample size permits.

VII. REFERENCES

Use, Maintenance, Performance Verification and Calibration of the FT-IR (LP 2075kk).

VIII. FORMS AND REPORTS-NA

VIII. DISTRIBUTION OF CONTROLLED COPIES

A. Originals of signed current versions of SOPs will be kept in the office of the Quality Manager

B. Copies of signed current versions of SOPs will be:
Distributed to appropriate personnel as determined by the author, Laboratory Supervisor and the Quality Manager

IX. ARCHIVING

A. The Laboratory Supervisor and Quality Manager should be notified of all SOPs that are eligible for retirement or revision

B. Retired SOPs and superceded versions of SOPs will be maintained as described in SOP 103 Document control.

X. SUMMARY OF REVISIONS

Date	Description of Change (include page/section information)
05/19/2011	Ethylene Glycol Kit referred to in SOP is discontinued. New kit is being utilized now.

Approved by: _____ Date: _____