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Abstract
Drug facilitated sexual assault (DFSA) is not a new phenomenon although it has recently become widely recognized. It occurs in incidences of sexual assault where the perpetrators have used drugs to debilitate their victims. Any substance that is administered to lower sexual inhibition and enhances the possibility of unwanted sexual intercourse is potentially a date rape drug.

Benzodiazepines are a common drug used as a tool in DFSA, mainly because they cause anterograde amnesia. They can be present in such low doses that they are often difficult to detect by conventional analytical techniques. Therefore, the goal of this project is to determine the usefulness of capillary electrophoresis electro-spray ionization time-of-flight mass spectrometry (CE-ESI-TOF-MS) for the detection of low dose benzodiazepines.

This will be carried out in three stages. The first stage will be to validate the operation of the CE system by using UV detection. Initially an ammonium formate buffer of high pH will be used to provide good separation and high electroosmotic flow (EOF). Later, experiments will be performed to develop a volatile buffer of low pH to assist in the ionization of the drugs via CE-MS. The pKa values for benzodiazepines are low (ca. 1.3-4.0), so they will be difficult to ionize. CE analyses at low pH may lead to improved sensitivity but long analysis time can occur when EOF is not present [1]. Therefore, the effect of additives such as β-cyclodextrin, polymeric coatings, as well as buffer pH, concentration and spray parameters will be examined to determine their ability to decrease analysis time while increasing efficiency and resolution.

Finally, the above methods will be applied to spiked urine samples and non-probative casework. Solid phase extraction (SPE) will be used prior to analysis to isolate the drugs from the matrix sample. This will be carried out in-line or as a separate step prior to the analysis via CE-MS.

Introduction
Benzodiazepines are an important class of drugs with a broad range of therapeutic effects, including sedative, hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant. Because of their wide usage, these depressants have the potential for interaction with other central nervous system depressants that can result in life-threatening or impaired situations [2].

Besides being used for therapeutic purposes, benzodiazepines are commonly associated with parties, nightclubs and raves because of their ability to cause euphoria and a drunk-like high. When taken in the presence of alcohol, the user experiences heightened effects as well as amnesia and unconsciousness.

These substances are easily slipped into a drink and may be consumed voluntarily or involuntarily by the victim. DFSA usually occurs when a victim unknowingly consumes a spiked beverage becoming incapacitated and helpless. The perpetrator can take advantage of the victim and use the effects of the drugs to aid robbery, assault and/or rape [16]. Studies have shown that alcohol is the most prevalent form of date rape incapacitation and often the effect of alcohol is combined with other substances such as benzodiazepines, GHB, ketamine or other illicit drugs.

Common low dose benzodiazepines include but are not limited to flunitrazepam, alprazolam, clonazepam, lorazepam and triazolam. Differences in the benzodiazepines are based on their structure and this may lead to changes in pharmacological effects. Benzodiazepines generally comprise a 1,4-diazepine ring with a benzene ring fused to carbons 6 and 7 and, typically, a phenyl group attached to carbon 5 [28]. Structural modifications are also possible.

They are preferred over barbiturates because at a given dose, the sedative effects of benzodiazepines are lower and there is less risk of dangerous effects such as deaths from overdoses. In addition, for certain benzodiazepines there is a tendency to produce anterograde amnesia where the victim remains somewhat alert and responsive during an attack but their memory of the events becomes vague and uncertain [18, 19].

The analysis of these benzodiazepines can be performed using different analytical techniques, which include an immunoassay analysis as a presumptive technique and high-performance liquid chromatography (HPLC) or gas chromatography mass spectrometry (GC-MS) as confirmatory techniques. These techniques detect these drugs at low ng/mL concentrations in both blood and urine [18, 28]. For example, doses of flunitrazepam of approximately 4mg can induce sedation with amnesia and will result in plasma levels of 2-3 ng/mL 24 hours later [24].

Background Information

Capillary electrophoresis, developed in the 1960s, utilizes fused silica capillaries to separate samples based on their size to charge ratio. A complete separation can be performed in a few minutes with excellent component resolution. Because of the small sample volumes required (a few nanoliters or less) CE is quickly becoming the analytical tool of choice for applications which are sample limited [20]. Samples are introduced into the capillary by applying pressure, vacuum or voltage through the capillary. The high field strengths used then move the injected species down the column due to electrophoretic and electroosmotic forces.

Electroosmotic flow is observed when an electric field is applied to a solution in a capillary that has fixed charges on its interior wall. In an uncoated fused silica capillary, silanol (Si-OH) groups attached to the interior wall of the capillary are ionized to negatively charged silanoate ($Si-O^-$) groups at pH values greater than three. Attracted to the negatively charged silanoate groups, the positively charged cations of the buffer solution will form two inner layers of cations (the diffuse double layer) on the capillary wall. The first layer is referred to as the fixed layer because it is held tightly to the silanoate groups. The outer layer, called the mobile layer, is farther from the silanoate groups. The mobile cation layer is pulled in the direction of the negatively charged cathode when an electric field is applied. Since these cations are solvated, the bulk buffer solution migrates with the mobile layer, causing the electroosmotic flow of the buffer solution. All ions (and uncharged species) will migrate in the same direction [20]. The rate of EOF is dependent on the field strength and the charge density of the capillary wall. The wall's charge density is proportional to the pH of the buffer solution. The electroosmotic flow will increase with pH until all of the available silanols lining the wall of the capillary are fully ionized.

The efficiency of capillary electrophoresis separations is typically much higher than the efficiency of other separation techniques like HPLC. However, unlike HPLC, in capillary electrophoresis there is no mass transfer between phases. In addition, the flow profile in EOF-driven systems is flat, rather than the rounded laminar flow profile characteristic of the pressure-driven flow in chromatography columns. As a result, EOF does not significantly contribute to band broadening as in pressure-driven chromatography. These separations can be optimized by a variety of means, including variation of buffer, electric field strength, temperature, and addition of chemical additives and organic modifiers [20].

As an organic modifier, a polymeric double coating has the ability to make the CE system more reproducible and quantifiable. It is based on the flushing of two solutions through the capillary. The first is the buffer containing the polycation, which sticks strongly to the capillary wall. The second is the running buffer containing the polyanion, which sticks to the first layer of the polycations forming a double layer. The polyanion contains sulfate groups and is insensitive to

pH variations. The excess negative charges create a high and reproducible EOF during a run, even at low pH. After each analysis, the coating can be stripped by rinsing with sodium hydroxide and water and the dynamic coating reapplied. This minimizes the wall adsorption effects and eliminates the inherent capillary inconsistencies experienced at low pH [1, 25].

CE analysis using chemical additives such as highly sulfated cyclodextrins produce improved resolution of a wide variety of drugs, particularly neutral compounds. At high pH there is a high EOF; however, at this high pH drugs such as benzodiazepines are neutral. Therefore, cyclodextrins, often represented as a core shaped cavity, are added to the buffer solution, the different benzodiazepines interact with the cyclodextrins to different extents, and separation is achieved [26]. EOF carries the benzodiazepines through the capillary and they arrive at the cathode before the negatively charged cyclodextrins.

Mass spectrometry is a destructive analytical technique that can reveal specific, characteristic and structurally related information about a compound based on its mass to charge ratio *(m/e)*, that is, the ratio of the mass of a given particle to the number of electrostatic charge units carried by the particle. It is generally used to find the composition of a physical sample by generating a mass spectrum representing the masses of sample components. The most important ion in the mass spectrum is the molecular ion. This is the peak of the highest abundance and is a direct indicator of the molecular weight of the compound. During the process of ionization, considerable excess energy may be transferred to the molecular ion, which, depending on its stability (related to its structure), may decompose into various fragment ions [21].

The operating principle of the time-of-flight (TOF) mass spectrometer involves measuring the time necessary for an ion to travel from the ion source to the detector. All the ions receive the same kinetic energy during acceleration, but separate into groups based on their different masses. The *m/e* value of an ion is determined by its time of arrival at the detector. Ions of low mass reach the detector before those of high mass since these heavier ions have a lower velocity. The resolving power of the TOF instrument is a function of flight tube length, accelerating voltage and, most important, spatial distribution of the ions [21]. The benefit of this instrument is that it has a high scan rate, a short analysis time and can determine the exact mass of each component in a sample to four decimal places.

In most laboratories, HPLC and gas chromatography (GC) have been established for the separation of various compounds [27] and GC coupled to mass spectrometry is the gold standard for forensic drug analysis. However, separations by HPLC show poor efficiency and a compound must possess certain properties to be GC compatible [17]. For example, many benzodiazepines are polar and non-volatile, making them difficult to analyze with GC or GC-MS [3]. While the laminar flow profile, slower mass transfer, and possible additional interactions with residual silanol groups limit separation efficiency in HPLC. As a result, high efficiency is generally obtained in CE because of a plug flow profile and faster mass transfer [27]. Recently, capillary electrophoresis has been proposed as an alternative for toxicological analysis, especially when coupled to electrospray mass spectrometry (ESI-MS). CE has several advantages that make it an ideal method to couple with ESI-MS. It has high efficiency, minimal sample requirements and short analysis time. This permits a wider variety of compounds to be analyzed than with standard HPLC or GC systems [22].

To obtain the identity of the sample capillary electrophoresis can be directly coupled to mass spectrometers using a sheath flow interface and electrospray ionization. In this system, the sheath liquid assists the elution and ionization of the analyte. The ions created are then analyzed by the mass spectrometer. The high resolution, sensitivity, and mass accuracy of ESI-TOF mass

spectrometers can provide practical applications in several areas and its ability to register ions of all masses in a single scan provides sensitivity over the full spectrum equal to that in the single ion-monitoring regime. The TOF also provides several advantages when used in combination with CE. The system is very fast compared to trap systems or quadrupoles and has a 3ppm or less mass resolution, enabling extremely accurate determination of compounds based on their masses. The system identifies drugs based on four operational parameters: absolute mass, prediction of related absolute isotopic mass abundances, in-source collisional dissociation, and electrophoretic mobility. The high separation efficiency of CE combined with the high sensitivity and informativeness of MS makes this instrument a powerful tool for screening and confirmation of drugs.

Research to Date

Few articles discuss the analysis of benzodiazepines by CE-MS or the combination of CE and TOF-MS, but many of them discuss ways in which the system can be optimized. The combination of analytical techniques such as CE, ESI and MS achieves optimum performance if each individual component operates with maximum proficiency, and is in full harmony or compatibility with the other components [4].

There have been articles published on the analysis of benzodiazepines with various techniques such as LC-MS [2, 3, 5] and GC-MS [6] as well as research on date rape drugs using HPLC-MS [6]. Within each of the techniques there have been areas where improvement can be made and, as a result, research is now moving in the direction of CE/MS.

Vanhoenacker et al were able to analyze benzodiazepines via CE-ESI-MS with a quadrupole ion trap using a low pH buffer and a double coating on the inner wall of the capillary. This procedure was then successfully applied to the analysis of a spiked urine sample after solid phase extraction [1]. McClean et al were also able to detect selected benzodiazepines with a quadrupole ion trap using a low pH buffer and the application of pressure during the separation step. By optimizing this method they were able to improve ionization efficiency significantly and ultimately lower the limits of detection [7]. Cherkaoui and Veuthey were able to separate anesthetic drug substances with a single quadrupole using a poly(vinyl alcohol) coated capillary, a buffer of low pH and negatively charged sulfated β–cyclodextrins. This study obtained high resolution and separation in less than twelve minutes.

With minor modifications to the methods carried out in these articles among others, specific parameters can be analyzed to determine the overall performance of the system with respect to its ability to perform rapid, reliable and sensitive analysis.

Proposed Methodology

- Validation and Separation of Benzodiazepines via CE System using UV detection

A number of low dose benzodiazepines as well as a few others used in DFSA will be dissolved in the appropriate amount of methanol to obtain a 1mg/mL concentration. These will then be serially diluted using the running buffer and analyzed at wavelengths between 195 and 280nm. Initially 20mM ammonium formate buffer at pH 8.0 will be used as the running buffer but this will later be replaced with lower pH 20mM formic acid buffer. The capillary column used will have a 50μm internal diameter and a length of 45cm. The effect of buffer pH, concentration, addition of additives such as β-cyclodextrin and polymeric EOF modifiers will be examined.

- Detection of Benzodiazepines via CE-MS

The above method will then be adapted for use with the mass spectrometer. These drugs will then be analyzed via this method using 20mM formic acid running buffer at pH 2.7. At this low pH, the drugs are assumed to be cationic and although a low EOF will result, electrophoretic mobility and induced flow resulting from the sheath liquid during analysis will enable overall decreased run times. Extracted ion analysis will be performed with high selectivity by using the exact masses of the protonated molecular ions. This capability greatly reduces background noise and improves detection. The capillary column used will have a 75μm internal diameter and a length of 95cm. When using the instrument, the polyimide coating will be removed from the end of the capillary and a blunt tip cut in the fused silica with a sapphire scribe. It is assumed that this is crucial when using the CE-MS to obtain a perfect and precise spray while eliminating adsorption of the sample on the outside of the capillary. The effect of buffer pH, concentration, addition of additives such as β-cyclodextrins and polymeric EOF modifiers will be examined again in addition to spray parameters. The results will demonstrate CE-ESI-TOF to be a rapid and highly specific detection method for benzodiazepines.

- Application of Method to Detect Benzodiazepines in Spiked Urine Samples

The importance of this study is its applicability to real life situations. In cases of DFSA, urine specimens are considered the specimen of choice for toxicological testing. This is because most drugs and metabolites are present at higher concentrations in urine and as a result, their presence can be detected for longer periods of time [18]. Therefore, urine samples will be obtained from individuals willing to participate in this study and these will be spiked in the lab. The drugs will be extracted from this matrix by solid phase extraction before analysis via CE-MS. In the future, it would be advantageous to use urine samples from individuals where DFSA was suspected. In this case, the metabolic processes the drug(s) go through in the body will become significant.

Significance of Study

Benzodiazepines are among the most commonly prescribed drugs. This increases their potential for diversion and abuse, and very often they are found in victims of DFSA [23]. Ingestion of these drugs often causes amnesia and unconsciousness. This loss in memory results in confusion and ultimately causes a delay in the notification of the authorities. This combined with the fast metabolism and elimination from the body results in only trace amounts of the parent drug being present in the victim's system. This makes the prosecution of such crimes more difficult. These concentrations are often below the detection limits of current instrumentation procedures and therefore require a method that is more sensitive.

For these reasons, the analysis of benzodiazepines is of great interest to forensic toxicologists. This study will provide a new approach to accurately and consistently determine the presence of benzodiazepines at low concentrations. In addition, due to high mass resolution, there is potential for very fast, accurate determination of a wide variety of compounds as well as being applied to cases where drug facilitated sexual assault has been suspected.

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