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Original Research Article

Determination of Asenapine Maleate from Maggots by Solid-Phase Extraction and Gas Chromatography - Mass Spectroscopy.

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Entomo-Toxicology, Entomological Fauna, Post Mortem Interval, Drug Detection.

Abstract

Introduction: Forensic entomology and Forensic entomo-toxicology are two fields which are interconnected and are creating a niche of their own in the area of research. In certain cases of drug abuse/ intoxication, when there are no viscera samples available for toxicological analysis, the fly larvae which feed the deceased flesh can be of great help in detection of the drug. In this process, environmental factors like temperature, rain, humidity etc. work as the variables, affecting the process. **Methodology**: Different concentrations of Asenapine maleate were spiked in five goat meat samples weighing 250 grams each, along with a blank control sample. They were then placed in an open environment to observe and study the natural decomposition process and the different life stages of the sarcophagus insects (flies). **Results and Discussion**: The drug was found in the maggots of samples containing 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml drug, but it was not found in the sample containing 10 µg/ml drug. The reason behind this is the limit of detection. **Conclusion**: It has been reported that different drugs are being abused regularly by humans for various reasons. Several cases have been reported wherein overdose of these drugs leads to death. In this study, an attempt was made to develop a protocol for detection and determination of drugs in relation with entomological specimens.

1. Introduction

The field of Entomo-toxicology was developed in 1980 by Beyer and his co-workers. They first used the insects feeding on the flesh of the deceased to identify the drug which was taken prior to death.[1] However, the field of forensic entomology was in use since long before for estimating the post mortem Interval (PMI). In China, a lawyer, Sung Tzu, who was also an

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investigator of death, conducted and documented the first case of forensic entomology in the 13th century. [2, 3] These two fields are completely based on the process of decomposition and the various factors affecting it. The process of decomposition of a body starts immediately after death. It is a process, and not an event, and it can be divided in to five different stages: i) fresh, ii) bloated, iii) decay, iv) post decay and v) skeletonization.[4] The rate of decomposition always depends on the external environment, exposure and other such conditions.

The decomposition rate automatically increases in the open, when the body is exposed to decomposers like flies, beetles etc.[5] Different species of flies are capable of laying different quantities of eggs on the corpse. These eggs grow into larvae which feed on the corpse and in a few days to weeks, it becomes skeletonised. Therefore, there will not be any tissues or blood left for toxicological analysis. In such cases, maggots found feeding on corpse are used as alternate specimens for toxicological analysis,[1] as they were actively feeding on the tissues which were carrying the drug.[6,7]

Drugs which are extracted from the decomposer species larvae are considered as evidence in the courts.[8] The development rate of maggots depends on many factors like environmental temperature, humidity level, drugs present in the tissues of the cadavers, etc. which sometimes increase or inhibit their development.[9-14] The larvae of calliphorid family and others show a great power of acceptance for drugs and drug induced tissues containing high dose of morphine,[15] barbiturates,[9] and amitriptyline,[16] in a dose which is considered lethal for an adult human. Significant development has occurred in the field of forensic entomo-toxicology.[17-19] In those countries where high temperature is generally observed, the use of insects for toxicological analysis is gaining much popularity. As the rate of decomposition increases with increase in temperature, the decomposition rate is high in these places. Hence sarcophagus insects and the field of entomo-toxicology are valuable for gathering evidence of drug related deaths.

Different techniques have been utilised for entomotoxicological analysis. These include gas chromatography, [20-23] liquid chromatography, [24-27] liquid chromatography mass spectroscopy and immunoassay.[28-30] Asenapine maleate [Saphris (3aRS,12bRS)-5-chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo (2,3:6,7) oxepino (4,5-c) pyrrole] is an atypical antipsychotic drug which can be used for the treatment of bipolar disorders and schizophrenia and is approved in the USA since 2009. This drug belongs to the dibenzo-oxepino pyrrole class and has a molecular weight of 285.8 g/mol. The empirical formula of asenapine maleate is $C_{17}H_{16}ClNO$.[31] It is a prescription drug and is generally prescribed as 5 and 10mg sublingual tablets. With a single 5 mg tablet, the adsorption of this drug occurs within 1.5 hours.The steady state of this drug is reached in 3 days. The metabolism of asenapine maleate occurs by glucuronidation and the oxidative metabolism through cytochrome P450 system in the liver. Its half-life is 24 hrs and about 50% can be recovered from urine and 40% recovery can be done from faeces. Common side effects of this drug include: drowsiness, restlessness, dizziness, numbing of mouth, weight gain etc. [30]

Asenapine maleate was reportedly involved in several suicide cases, according to a report by National Center for Biotechnology Information (NCBI).[32] Information regarding the analysis of asenapine maleate from visceral samples is limited. Researchers performed the analysis of asenapine with solid-phase extraction (SPE) or liquid-liquid extraction followed by high-performance liquid chromatography (HPLC), liquid chromatography with tendem mass spectrometry (LC-MS-MS) and Gas chromatography mass spectrometry (GC-MS). [33,34]

2. Materials and Methodology:

Apparatus - A total of 6 jars of 1kg capacity each, were used for keeping the meat substrates. Six boneless goat meat pieces weighing 250gms each, were purchased from a butcher shop in Sector 67 Mohali, India.

Reagents – The drug asenapine maleate was purchased from Indian Pharmacopeia with 99.9 percent purity. The methanol was HPLC grade from Qualigens. Liquor ammonia, 25%V/V was purchased from Fisher Scientific.

Physical and Chemical properties of the drug - Asenapine maleate is soluble in water and methanol. The pka of the drug is 7.24. It is a non-hygroscopic off-white powder with a boiling pointof 357.9ºC at 760 mmHg and melting pointof 141-145°C. Flash point is 170.2ºC. Its molecular formula is $C_{21}H_{20}ClNO_5$ and molecular weight is 401.84.[35]

Sample Treatment

A. Stock solution was prepared by dissolving 10 mg asenapine maleate in 10 ml methanol of HPLC grade resulting in a solution of 1mg/1ml w/v.
B. Working solutions of 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50 µg/ml were prepared by using solvent methanol for tuning of mass spectrometry and selectivity of experiment.

All the working solutions and stock solution were stored at -20^0 C till use.

Geographical and environmental conditions - The experiment was performed on location NL 30◦45'51" EL 76◦37'40" in Mohali city of Punjab, India. The location is situated in Malwa region of Punjab. The minimum temperature during experiment was 4^0 C and the maximum temperature was 24^{0}C, with an average temperature of 12^0 C. The average humidity was 89% during the experiment. The experiment was set up in an open garden area of a rented house in sector 115, Mohali, Punjab.

Sample preparation - Different spiked drug concentrations were injected in to the meat samples in the ratio of 10, 20, 30, 40, 50 µg/ml each, excluding the control, respectively. Then, these five different concentrations and the blank control entomo-toxicological samples were kept in open for observation of the natural decomposition process.

Sample collection - The process of decomposition was observed carefully and each life stage of insects was recorded through photographs. The larvae of the feeding and post feeding stage were collected after an interval of every 8 hours; a total of 50 larvae from each concentration of the drug and the blank sample. For the purpose of cleaning, larvae were washed and rinsed with water to avoid the surface drug amount from detection through the instrument as an artefact. After washing, the larvae were stored in air tight containers and preserved in the freezer at -10^{0}C till analysis.

Figure 1: Samples placed in different jars named A, B, C, D, E & F

Figure 2: Various Stages from development (Egg deposition, maggot formation, pupa & adult fly).

Isolation, cleaning-up, extraction and purification procedures - Maggots of each concentration were first minced using surgical blade and placed in Borosil® beakers separately. After that, 5 ml methanol was added to all the maggot samples. The pH was adjusted to 9 using liquid ammonia as the best extract was found in pH 9. Solvent pH was checked using pH strip. After checking pH, mixture was filtered using filter paper. (Whatman®125mm). The filtrate was then vortexed and centrifuged at 2500 rpm for 5 minutes. The clear liquid obtained after centrifugation was used for solid-phase extraction.

Solid-phase extraction - SPE instrument used was the offline Superclean Ultra 2400 model with reversed phase discovery c8 column. The clear liquid was poured through the cartridge and proper vacuum was maintained during extraction. The extract was then poured in a china dish and covered using aluminium foil after making holes using a pin and was left for 24 hours.

Instrumentation

Gas chromatography (GC) - Routine analysis and separation of commonly using psychotropic substances, narcotic drugs and pesticides were done with an auto-sampler Shimadzu AOC-20N Plus coupled to a GC–MS Shimadzu QP-2020 NX (Kyoto, Japan) equipped with a split/split less injector in the split-less mode using a SH- RXi-5Sil-MS fused silica capillary column of 30 m×0.25 mm ID×0.25 µm stationary film thickness, manufactured by Shimadzu (made in USA) with the following conditions: constant flow of Helium (He) 1.2 mL/min; initial inlet temperature 90°C ramped to 290°C at 200°C /min after a 30sec delay; injection volume 5µL (LVI) onto a Carbofrit plug in the liner with an open purge valve

(30:1 split ratio) for 10s, closed until 3.0 min and open again (30:1) until the end of the run; oven temperature program: 85°C for 3 min then25°C /min ramp to 180°C followed by a 10°C /min ramp to 300°C and held for 4 min. The total time for one GC-MS run was 23 min.

Mass spectrometry (MS) - The MS instrument transfer line temperature was 240°C; ion source temperature 230 °C; ionization mode – electron impact at 70 eV full-scans (30–550 m/z). The optimization of the retention times and chromatographic resolution were done in the scan mode with all prepared standard concentrations.

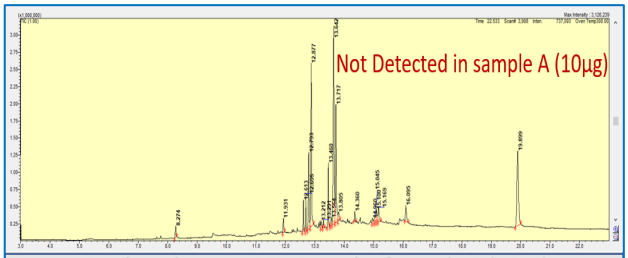
3. Results and Discussion:

The drug was found in the maggots' samples containing 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml; but it was not found in the sample containing10µg/ml drug (**table 1 and Graph 1 to 6**). The reason behind this is the limit of detection. The drug in the quantity 10µg/ml is usually not detectable as it is below the limit of detection. The drug was also not detected in maggots of the Blank sample, used as control sample. This particular drug was not found in literature survey used for entomo-toxicological studies. By using this drug, a new protocol for the detection of this drug has been made and it can be used in future cases related to this particular drug. This drug is reportedly abused and involved in many deaths.[35] Maggots are compatible for qualitative analysis of drug and in future quantitative analysis can be the focus of research. This drug was previously used in different studies in which asenapine was spiked in human plasma and urine. The extraction was done using automated solid phase extraction and analysed using HPLC-MS/ MS and liquid chromatography-tandem mass spectrometry [33,34] whereas in present study the drug was spiked in meat substrate and extracted from maggots by using solid phase extraction and analysed through GCMS. In another study, nineteen different drugs were spiked in tissue, larvae and hairs. The analysis was done using GCMS and chemiluminescence method but the drugs were analysed in tissue and hairs but drugs were rarely detected in maggots [36] whereas in present study drug detection was done more accurately. There was a study done for nicotine detection through maggots using liquid-liquid extraction and analysed using GCMS but in present research, different extraction technique was used and lower detection limit was gained.[37]

Table 1: Results found in different samples carrying different concentrations of drug.

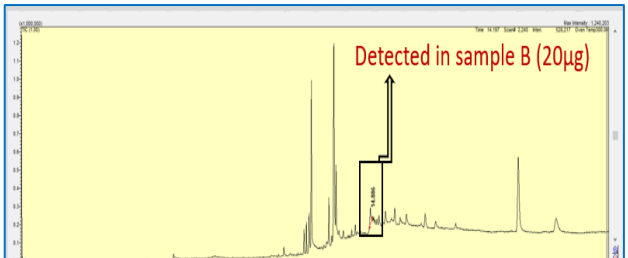
Samples no.	Drug Concentration	Detection
A	10µg/ml	Not detected
B	20µg/ml	Detected
C	30µg/ml	Detected
D	40µg/ml	Detected
E	50µg/ml	Detected

Graph 1: GCMS Graph of sample A (10 ug)



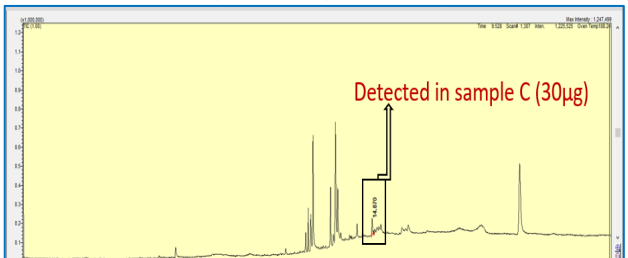
Graph 1. showing GCMS graph of sample A (10µg)

Graph 2: GCMS Graph of sample B (20 ug)



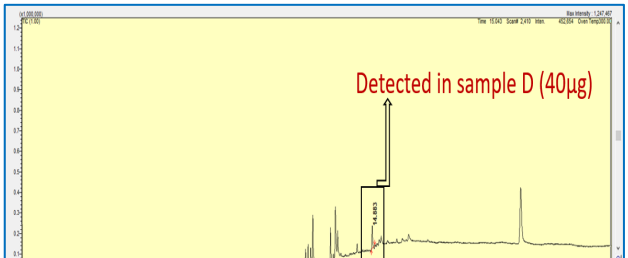
Graph 2. showing GCMS graph of sample B (20µg)

Graph 3: GCMS Graph of sample C (30 ug)



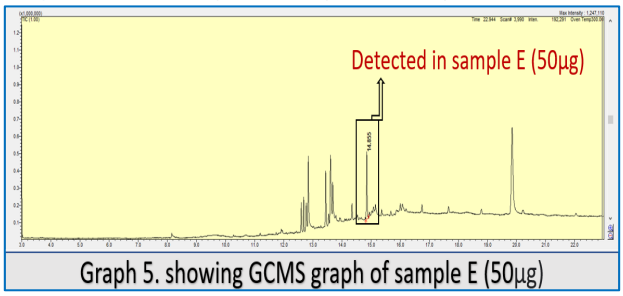
Graph 3. showing GCMS graph of sample C (30µg)

Graph 4: GCMS Graph of sample D (40 ug)



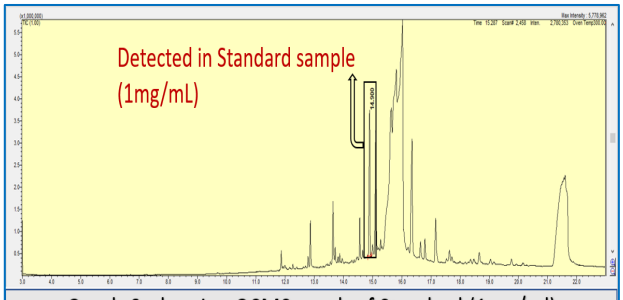
Graph 4. showing GCMS graph of sample D (40µg)

Graph 5: GCMS Graph of sample E (50 ug)



Graph 5. showing GCMS graph of sample E (50µg)

Graph 6: GCMS Graph of standard sample (1 MG/ML)



Graph 6. showing GCMS graph of Standard (1mg/ml)

5. Conclusion

According to this research, Asenapine maleate, a drug that has been linked to numerous cases of drug abuse and death, can be detected through the larvae, even when the visceral organs are not available for drug analysis. Insects found at a crime scene can be helpful in estimating the time since death by observing their growth stage, but the effect of drugs on the life cycle of insects can alter the estimation of post-mortem interval (PMI). Since different drugs have different effects on different insects, further studies should be carried out using different drugs and insect species to improve knowledge in this field. This would allow investigators to narrow down the pool of suspects. In addition, future entomo-toxicological studies should focus on documenting a detailed summary of drugs and their correlation with insect species, as well as quantifying drugs in these specimens.

The findings of this study are significant because they offer an alternative way to detect the presence of Asenapine maleate in the absence of visceral organs. While insects found on crime scenes have traditionally been used to estimate PMI, the presence of drugs can alter the life cycle of insects and complicate these estimations. Therefore, it is important to understand the effects of drugs on different insect species, which would help investigators to better estimate PMI and narrow down the pool of suspects.

Ethical Clearance: IEC approval is taken from the Institutional Ethical committee.

Contributor ship of Author: All authors equally contributed.

Conflict of interest: None to declare.

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