The Determination of Morphine in the Larvae of Calliphora stygia using Flow Injection Analysis and HPLC with Chemiluminescence Detection*

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Abstract

Selective determination of morphine in the larvae of Calliphora stygia (Fabricius) (Diptera: Calliphoridae) using acidic potassium permanganate chemiluminescence detection coupled with flow injection analysis and high-performance liquid chromatography (HPLC) is described. Larvae of C. stygia were reared on minced meat substrates that had been spiked with varying concentrations of morphine. Morphine concentrations were chosen to reflect typical levels in human tissues from opiate overdose victims. After maturing on substrates, larvae were analyzed for the presence of morphine using chemiluminescence detection coupled to flow injection analysis and a rapid HPLC method. Analysis of the larval matrix by flow injection analysis with chemiluminescence detection indicated the presence of interferants capable of generating chemiluminescence. A rapid chromatographic separation with a monolithic column allowed selective determination of morphine in larvae using postcolumn chemiluminescence detection. Larvae of C. stygia reared on substrates containing morphine at concentrations of 500 and 1000 ng/g did not sequester morphine at detectable concentrations. Larvae reared on substrates containing morphine concentrations of 2500, 5000, and 10,000 ng/g tested positive for the drug at concentrations of 765, 2720, and 3010 ng/g, respectively.

Introduction

Forensic entomology applies the study of insects to the investigation of legal problems. One of the most common uses of forensic entomology is the aging of dead bodies using carrion insect larval growth and predictable carrion insect species composition changes as a timeline. An emerging branch of forensic entomology, known as entomotoxicology, exploits flesh feeding insects as alternative toxicological specimens because they bio-accumulate drugs and toxins. This is especially useful in the later stages of decomposition because traditional toxicological samples, such as soft tissue, blood, or urine, will no longer be available from the body, whereas insects may be present for many months after death.

Research in entomotoxicology has so far produced a number of analytical protocols for using insects as toxicological specimens in case work (1). Early work by Beyer et al. (2) detected phenobarbital in the larvae of the blow fly Cochliomyia macellaria (Fabricius) (Diptera: Calliphoridae), which were feeding on heavily decomposed human remains. Phenobarbital was detected using gas chromatography (GC) and thin-layer chromatography as a confirmatory test. Subsequently, Nuorteva and Nuorteva (3) described a method for determining mercury from various species of blow fly larvae (family Calliphoridae), indicating bio-accumulation of such heavy metals in the maturing larvae feeding on the contaminated tissues. Leclercq and Brah y (4) detected the presence of arsenic in larvae of the fly families Piophilidae, Psychodidae, and Muscidae. The successful detection of the organophosphate insecticide malathion in calliphorid larvae using GC was reported by Gunatilake and Goff (5). Toxicological analysis of the insect larvae corroborated the results of tissue analysis to identify malathion poisoning as the cause of death, which further supports the potential use of fly larvae as alternative toxicological specimens.

Since this pioneering work, several groups have successfully employed entomotoxicological analysis, combined with corroborating tissue analysis, for a variety of other toxicologically important substances. New analytical methods have also been used for the determination of analytes detected by earlier workers. Such methods include high-performance liquid chromatography (HPLC) (for the detection of triazolam, oxazepam, phenobarbital, alimemazine, and clomipramine) (6), GC (for...
therefore, isolation and detection of morphine, especially from a steady increase in the number of heroin overdoses (11); toxicologists. It can be wholly or partially responsible for challenging matrices, is an ongoing research priority. It was causing death, and occurs in bodies following antemortem ingestion of heroin and morphine-based pain killers, sedatives, or anxiolytics (10). Over the past 20 years there has been a steady increase in the number of heroin overdoses (11); therefore, isolation and detection of morphine, especially from challenging matrices, is an ongoing research priority. It was therefore chosen as the analyte of choice in the present study. It is additionally suitable for study because of its stability in biological matrices, including toxicological samples of both human and insect origin. 

Chemiluminescence is the product of a chemical reaction that yields an electronically excited species, which emits light as it returns to the ground state. Excess energy produced from a chemical reaction is usually lost in the form of heat due to collisional vibrations. However, chemiluminescent reactions lose a portion of this excess energy through the emission of photons, ranging from the near ultraviolet to the near infrared (12,13). Chemiluminescence has a number of inherent advantages over other photoelectromagnetic methods in that it offers excellent selectivity and does not require an electromagnetic excitation source, therefore allowing very low limits of detection (14). Instrumentation required for the implementation of chemiluminescence detection is both cost effective and robust, making it more analytically appealing than other photoelectromagnetic methods. The selectivity of certain chemiluminescent reactions has been employed for the detection of various analytes in complex matrices without any prior separation (15–18).

Potassium permanganate is a common oxidant used widely in analytical chemistry as a reagent to generate chemiluminescence during the oxidation of both organic compounds, and inorganic species (19). Acidic potassium permanganate exhibits strong chemiluminescence on reaction with morphine and offers improved selectivity and sensitivity when compared to UV absorbance detection. Selectivity is achieved by a combination of the extreme selectivity of the reaction and the dilution of any interferants present in the matrix, which may be capable of generating additional chemiluminescent responses when oxidized by the reagent (15–18). Although no experimental data was generated using UV-vis detection, its performance was evaluated and proved far less sensitive than chemiluminescence detection. Because of the low expected concentrations of morphine in larvae, potassium permanganate chemiluminescence was employed over the more traditional UV-vis detection method to achieve lower limits of detection. 

The native Australian carrion blowfly Calliphora stygia Fabricius (Diptera: Calliphoridae) was also used in this study. This species colonizes bodies year round in early decomposition and is common in southeastern Australian and New Zealand forensic cases (Dr. Melanie Archer, personal communication).

The present study describes a sensitive and selective method for the determination of morphine in the larvae of C. stygia. Chemiluminescence detection coupled with flow injection analysis or HPLC was used in all experiments.

Materials and Methods

Blow fly cultures

Cultures of C. stygia were maintained at uncontrolled room temperature between March and October in a 12 h light/12 h dark cycle. Colonies were replaced with wild-type individuals approximately every two months to avoid potential inbreeding effects.

Substrate preparation and larva rearing

Eggs were transferred to rearing substrates (approximately 50 eggs per substrate) and left to hatch. Experimental rearing substrates consisted of 100-g portions of minced beef containing standardized morphine concentrations chosen to reflect the upper and lower concentration limits commonly encountered in autopsy samples (10). Morphine solutions were homogenously distributed in the meat substrate using a mortar and pestle. Six experimental substrates were prepared with morphine concentrations of 0 (control), 50, 100, 2500, 5000, and 10,000 ng/g using morphine solutions prepared from a fresh stock solution (1.0 × 10⁻³ M). HPLC coupled with postcolumn chemiluminescence detection was employed to analyze five sub-samples from each substrate to ensure that acceptable morphine concentrations were distributed throughout the substrates.

Sample preparation

Following maturation to the prepupal stage, all larvae (approximately 50) were removed from the food source, thoroughly washed with distilled water, and dried using paper towel. This process was done in triplicate to reduce the possibility of surface contamination. Dried larvae were counted into five groups, weighed, macerated, and homogenized. Following preparation, 0.5 g of homogenate was weighed and combined with 5 mL of acetic acid (1%) to extract any morphine from the insect matrix. Samples were then sonicated to liberate the analyte and centrifuged at 3500 rpm for 15 min (15°C). Following centrifugation, duplicate samples of the supernatant were analyzed for morphine.

Flow injection analysis with chemiluminescence detection

Reagents and carrier solutions were propelled using a peristaltic pump (Gilson Minipulse 3, John Morris Scientific, Australia) through bridged PVC tubing (1-mm i.d., PROTECH,
HPLC with chemiluminescence detection

HPLC separations were performed using a Hewlett-Packard series 1100 LC system (Agilent Technologies, Australia) equipped with both UV-visible detection (Hewlett-Packard, Australia) and a Hewlett-Packard analogue to digital interface box for analogue input from the chemiluminescence detector. Separations of morphine from matrix interferents were accomplished using a Chromolith™ SpeedROD RP-18e (50 × 4.6-mm i.d (Merck KgaA, Germany) and an injection volume of 5 μL was used throughout all analyses. The column eluate and chemiluminescent reagent merged at a T-piece located approximately 15 mm from a spiral flow through cell which was mounted flush against the window of a red sensitive photomultiplier tube (Thorn-EMI model 9924BS, ETP, Ltd., Australia). Detection systems were operated at a constant voltage of 900 V provided by a stable power supply (Thorn-EMI model PM28BN, ETP, Ltd.). Flow cell, PMT and voltage divider were all encased in light tight housing. Output from the photomultiplier tube (PMT) was documented using a chart recorder (YEM Type 3066, Yokogawa Hokushin Electric, Tokyo, Japan).

Acidic potassium permanganate chemiluminescence

All reagents were of analytical grade unless otherwise stated and solutions were prepared with deionized water (Millipore, MilliQ Water System, Bedford, MA). Potassium permanganate (Ajax, Sydney, Australia) solutions (5.0 × 10⁻⁴ M) were prepared daily in sodium polyphosphate (Aldrich, Milwaukee, WI) solution (1%, w/v, pH 2.5), and adjusted to pH 2.5 using phosphoric acid (Ajax). All mobile phases were filtered through a 0.45-μm membrane filter prior to use. Aqueous stock solutions of morphine (1.0 × 10⁻³ M, GlaxoSmithKline, Port Fairy, Australia) were prepared daily in deionized water and diluted as required. All morphine extractions were completed using 1% acetic acid (Ajax).

Results and Discussion

Preliminary FIA results

Fifty control samples of maggot matrix were prepared and analyzed independently using flow injection analysis to detect biological interferants capable of producing chemiluminescence in the presence of acidic potassium permanganate. Prior to analysis, five samples of all rearing substrates were also analyzed to ensure that any matrix interferences in the larvae did not originate from the food source. Rearing substrates exhibited no chemiluminescent response when reacted with acidic potassium permanganate. Under the same conditions, the maggot matrix elicited a large chemiluminescent signal (Table I) indicating that sample cleanup was required in order to eliminate false-positive detection.

Sample cleanup

Previous investigations have found that proteins and amino acids generate a significant chemiluminescent response when oxidized by acidic potassium permanganate (20–22). Therefore, several experiments were performed in an attempt to remove large molecules, such as proteins and lipids from the sample matrix (Table I).

Unfortunately, the level of sample clean up achieved in preliminary experiments was not sufficient to allow low limits of detection. Therefore, further separation of the matrix was necessary to ensure the selective determination of morphine at low levels. HPLC was employed for the remainder of the project to separate morphine from the matrix interferants.

Instrument validation

A typical external standard method was used to obtain the calibration curve that was subsequently used to quantify all morphine responses in HPLC experiments. Five aqueous morphine standards were prepared over the concentration range 2 × 10⁻⁶ M to 1.0 × 10⁻⁵ M. The resultant calibration function had a linear correlation coefficient value (R²) of 0.9959.

Analysis of morphine content in rearing substrates

Analysis of rearing substrates was performed using HPLC coupled with post-column chemiluminescence detection to ensure accurate spiking of substrates. All substrates containing morphine tested
were reared on substrates containing the opiate, may have sequestered the drug at concentrations lower than the limits of detection for this methodology. However, the recovery rate increased only slightly for the substrate containing 10,000 ng/g (Table III).

Larvae were reared on five different 100-g minced meat substrates containing varying concentrations of morphine, and one control substrate with no morphine (Table III). Successful determination of morphine was achieved in larvae reared on substrates containing morphine at concentrations of 2500, 5000, and 10,000 ng/g. Five homogenates of larvae reared on substrates containing 500, 1000, and 0 ng/g of morphine tested negative for the drug (Table III). Limit of detection (signal-to-noise ratio = 3) for this methodology was 2500 ng/g of morphine.

Morphine recovery rates from maggots feeding on tissues with known morphine concentrations increased appreciably between substrates containing 2500 and 5000 ng/g. However, the recovery rate increased only slightly for the substrate containing 10,000 ng/g (Table III).

Larvae that tested negative for the presence of morphine, but were reared on substrates containing the opiate, may have sequestered the drug at concentrations lower than the limits of detection for this methodology. However results indicate that morphine can be successfully detected in the larvae of *C. stygia* at concentrations at and above 2500 ng/g. Successful detection of morphine in larvae reared on substrates S3, S4, and S5 further supports the potential for using carrion-feeding insects as alternate toxicological specimens. The methodology developed here is not only simple, but also rapid and robust.

No significant statistical correlation was observed between morphine concentrations in rearing substrates and those sequestered in larvae. Although no statistical relationship was identified, it was evident that sequestered morphine concentrations in larvae increased with increasing substrate concentrations. This indicates that sample sizes for each replicate must be greatly increased to investigate the nature of any potential statistical relationship between tissue and maggot concentrations.

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### References


