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ORIGINAL ARTICLE

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Drug analysis in blowfly larvae and in human tissues: a comparative study

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Abstract The present study investigates the correlation between concentrations of drugs in human tissues and Diptera larvae feeding on these tissues. Samples of liver were taken from 18 cases in which preliminary toxicological screening indicated the presence of drugs. Blowfly larvae (Diptera: Calliphoridae) were reared on these samples and subsequently analyzed for drug content. Toxicological analyses were carried out using ONLINE Abuscreen (Roche) and GC/MS for available body fluids (blood, urine and bile) as well as liver samples and maggots. All drugs detected in human tissues were also detected in insect specimens. Opiates, cocaine and barbiturates as well as some antidepressants (clomipramine, amitryptiline, nortryptiline, levomepromezine and tioridazine) were observed. Comparisons of drug concentrations between those in human tissues and blowfly larvae showed different patterns of distribution that may be attributed to differences in physiology. Results confirm the reliability of entomological specimens for qualitative analyses, although quantitative extrapolations are unreliable. All xenobiotics detected were in higher concentrations in human tissues than in maggots. Concentrations in post-feeding maggots were significantly lower than for feeding maggots, suggesting that the feeding state of maggots may affect toxicological analyses as they metabolize and eliminate drugs during development.

Keywords Forensic entomology · Entomotoxicology · Drug analysis · GS/MS · *Lucilia sericata* (Diptera: Calliphoridae)

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Introduction

For badly decomposed bodies, fly larvae can be used as a substrate for toxicological analysis in addition to providing data concerning the postmortem interval when poisoning is suspected. Diptera larvae are often found on decomposing bodies long after tissues traditionally sampled for toxicological analyses, such as blood, urine or solid organs, have disappeared. Beyer et al. (1980) first published this technique, identifying phenobarbital in maggots associated with a body where no tissues suitable for analysis were present. These findings supported a final diagnosis of death by drug intoxication. Diptera larvae feeding on tissues from an individual who had taken drugs while still alive, ingest these substances as well as their metabolites. Bourel et al. (2001) recently demonstrated drug accumulation inside such feeding maggots using immunohistochemical techniques. In this paper, the rapid absorption of morphine present in human tissues through the intestinal epithelium of the maggots was demonstrated along with subsequent deposition of the morphine in an area lying between the endocuticle and exocuticle.

The toxicological analyses used for insect materials are generally the same as those used for human tissues and biological fluids (Goff and Lord 1994; Introna et al. 2001; Gagliano Candela and Aventaggiato 2001) and include radioimmunoassay (RIA), gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography-mass spectrometry (HPLC-MS).

Previous studies have demonstrated that most of the substances involved in drug-related deaths are also detectable through analyses of maggots feeding on the corpse: opiates such as morphine and codeine (Introna et al. 1990; Goff et al. 1991; Kintz et al. 1994; Hédouin et al. 1999), cocaine and benzoylecognine (Goff et al. 1989; Manhoff et al. 1991; Nolte et al. 1992), amphetamines (Goff et al. 1992, 1997), tricyclic antidepressants (Wilson et al. 1993; Goff et al. 1993; Miller et al. 1994; Sadler et al. 1995, 1997a), phenotiazines and benzodiazepines (Kintz et al. 1990a, 1990b; Sadler et al. 1997b; Carvalho et al. 2001)

as well as barbiturates and several salicylates (Beyer et al. 1980; Kintz et al. 1990c; Sadler et al. 1997b). Drugs and toxins have also been detected through analyses of developing blowfly larvae and empty puparial cases (Goff et al. 1993) and even insect fecal material (Miller et al. 1994).

To date no reliable correlation has been demonstrated between concentrations of drugs and/or toxins in larvae and the tissues on which the specimens had fed. Several earlier studies suggested such a correlation might exist, particularly for opiates and cocaine (Goff et al. 1989, 1991; Introna et al. 1990; Kintz et al. 1990b), while other studies showed no relevant correlation (Kintz et al. 1990a, 1994; Nolte et al. 1992; Sadler et al. 1997a, 1997d). In all of the previous studies, it was observed that the concentration of the drug or toxin was significantly lower in the maggots analyzed than in the tissues used as a food source. As was noted by several workers (Introna et al. 1990; Sadler et al. 1995. 1997c; Hédouin et al. 1999) the insects metabolize and eliminate the ingested substances during their development. The wide variations demonstrated in the various studies cited above show that the accumulation of a drug by larvae is unpredictable and quantitative extrapolations are unreliable (Sadler et al. 1995, 1997a). This is particularly true when dealing with more than a single drug or concentration level (Sadler et al. 1997b, 1997c). While Diptera larvae are useful as qualitative toxicological specimens, they are of limited quantitative value.

The present study is a comparison of drug analyses of maggots and human tissues from 18 bodies in which a preliminary drug screening of body fluids tested positive for narcotic intoxication and for which the cause of death was determined to be drug-related.

Materials and methods

A series of 18 drug-related necropsies has been selected. Most of the bodies were fresh and well preserved with a postmortem interval (PMI) <24–36 h; only one corpse was partially skeletonized (PMI 4–5 days) with very little soft tissue still present due to the action of local macrofauna and Diptera larvae. A preliminary drug screening was made on the available tissues, including body fluids (blood, urine and bile) or liver tissue samples from each of the 18 drug-related deaths by the EMIT system as well as GC/MS to provide qualitative results. The immunochemical ONLINE Abuscreen technique was also used. This analytical procedure guarantees a high sensitivity (ng/ml) and, as no extraction is needed, is based directly on biological substrates. However, since ONLINE Abuscreen is only suitable for qualitative and semiquantitative analyses of opiates and cocaine metabolites, quantitative analyses were also conducted using routine GC/MS procedures. Preparation and extraction methods for human tissues (body fluids and liver samples) were the same as for the blowfly maggots artificially reared on the drug-spiked liver tissue. Only for the single pre-skeletonized corpse, no rearing was performed since Diptera larvae collected directly from the remnants of liver tissue still present were considered suitable for toxicological analyses.

Liver samples were available and analyzed for each of the 18 bodies. Larvae of *Lucilia sericata* (Diptera: Calliphoridae) were reared on spiked liver tissue (except for the pre-skeletonized body) and both active feeding mature maggots and post-feeding maggots were collected. Fresh greenbottle (*Lucilia sericata*) eggs were transferred onto spiked liver samples, reared in an incubator at 25–28°C, 70–80% humidity and with cyclical artificial lighting simulating 16 h daylight and 8 h darkness. Further fresh portions of thawed liver were introduced as required. Mature larvae were collected at their peak feeding period after 4 days from oviposition while post-feeding larvae were collected after 6 days from oviposition. Once removed from the food source, maggots were washed with deionized water and stored at –20ºC. Prior to analyses, all specimens were again washed with deionized water, dried and then homogenized with deionized water (ratio 1:5) to eliminate surface contamination (Sadler et al. 1997d). Samples of human tissues were also homogenized. Following incubation for 48 h, 1 g of homogenate was centrifugated and the supernatant tested by using ONLINE Abuscreen for opiates and cocaine metabolites. GC-MS was performed on a Hewlett Packard 6890 series gas chromatograph equipped with a Hewlett Packard 5973 mass selective detector. Separation was performed on a 15m×0.25 mm I.D. HP-5MS fused-silica capillary column (95% dimethyl, 5% diphenyl polysiloxane) with a 0.25 µm film thickness and with helium as the carrier gas at a constant flow-rate of 1 ml/min. The splitless mode was used with 1 µl samples being injected. The operating conditions for the analyses were: injection port temperature 270°C, initial temperature 70°C for 2 min, programming 40°C/min to 160°C and 8°C/min to a final temperature of 290°C for 1.5 min.

Immunochemical results were confirmed with GC/MS in the SIM mode following solid phase extraction (Bakerbond C18 500 mg), derivatization with BSTFA at 70°C for 15 min and selected ions: 236, 287, 429 m/z for morphine-2TMS and 82, 240, 361 m/z for benzoylecgonine-TMS. For phenobarbital quantification, the internal standard (butalbital) was added to the biological samples and extracted with the liquid-liquid technique. The extracts were analyzed by GC/MS in the SIM mode after flash-alkylation with MethElute and selections of the ions 232, 260 m/z for methylated phenobarbital and 195, 196 m/z for methylated-ISTD.

Also tioridazine, clomipramine, amitryptiline, nortryptiline and levomepromazine were detected with GC/MS in the SIM mode following liquid-liquid extraction directly applied to biological samples with SKF 525-A added as internal standard and selected ions 58, 202 m/z for amitryptiline, 202, 263 m/z for nortryptiline, 98, 370 m/z for tioridazine, 58, 328 m/z for levomepromazine, 58, 269 m/z for clomipramine and 86, 165 m/z for SKF 525-A.

Results and discussion

Analyses performed on human tissues and maggots reared on spiked liver samples demonstrated the presence of the most common narcotic agents, such as opiates and cocaine, frequently mixed, as well as the antidepressants (clomipramine, amitryptiline, nortryptiline, levomepromazine, and tioridazine) and benzodiazepines, such as phenobarbital. All drugs detected in samples of human tissues were also detected in reared maggots (Tables 1, 2, and 3) and in those specimens collected from the pre-skeletonized body (case 15). Opiates were detected in 14 cases (Table 1) and in 8 out of the 14 cases, cocaine was also present (cases 1–4, 6, 9, 10, 14). In only one case were opiates and cocaine combined with phenobarbital (case 6). As shown in Table 1, there were different concentrations of opiates in different body tissues. In particular, concentrations of opiates in liver tissues were significantly higher than those for blood samples and in all maggots analyzed, both feeding and post-feeding.

Concentrations in maggots were also significantly lower than those observed in blood samples. This is consistent with the metabolism and elimination of drugs by maggots during development. In 4 cases, opiate concentrations in feeding maggots were slightly higher than concentrations

Table 1 Opiate concentrations (μg/ml) in morphine equivalents

Cases	Blood	Urine	Bile	Liver	FL	PFL
[a]1	0.72	26.75	103.50	2.45	0.75	0.14
[a]2	2.21	0.39	3.60	2.70	2.03	0.39
[a]3	0.85	73.50	287.50	0.95	0.63	0.42
[a]4	0.48	–	1.45	1.00	0.65	0.37
5	0.65	23.25	0.65	0.79	0.31	0.18
[b]6	0.59	0.13	0.92	1.52	0.48	0.20
7	1.57	9.95	5.85	3.07	0.29	0.21
8	1.86	19.70	22.20	3.20	1.16	0.37
[a]9	1.09	59.50	6.70	3.97	0.53	0.39
[a]10	0.60	3.44	–	1.86	1.30	0.30
11	0.27	–	29.75	1.19	0.30	0.30
12	0.52	0.54	13.90	0.71	0.30	0.26
13	–	27.60	35.50	7.05	0.31	0.22
[a]14	1.40	–	3.32	2.06	0.40	0.13

[a]Positive analysis for opiates and cocaine.
[b]Positive analysis for opiates, cocaine and phenobarbital.
– Sample not available.
FL Feeding larvae.
PFL Post-feeding larvae.

Tables 2 Cocaine concentrations (μg/ml) in benzoilecgonine equivalents

Cases	Blood	Urine	Bile	Liver	FL	PFL
[a]1	1.34	38.75	65.50	3.30	1.99	0.40
[a]2	1.60	0.18	7.55	1.63	1.62	0.37
[a]3	0.14	0.42	0.49	0.95	1.01	0.82
[a]4	0.64	–	43.50	1.92	1.92	0.71
[b]6	2.58	33.00	10.80	3.17	1.27	0.32
[a]9	0.50	89.50	7.65	4.55	0.62	0.57
[a]10	0.65	1.80	–	0.45	0.59	0.30
[a]14	1.60	–	5.15	1.82	0.48	0.47

[a]Positive analysis for opiates and cocaine.
[b]Positive analysis for opiates, cocaine and phenobarbital.
– Sample not available.
FL Feeding larvae.
PFL Post-feeding larvae.

Table 3 Prescription drug concentrations (μg/ml)

Cases	Drug	Blood	Urine	Bile	Liver	FL	PFL
[b]6	Phenobarbital	15	14.90	36.80	32.70	1.70	5.20
15	Phenobarbital	–	–	–	241.50	28.37	35.40
16	Levopromazine	0.01	0.12	0.53	0.29	0.04	0.06
17	Amitriptyline	0.40	0.56	13.16	9.42	0.65	0.01
	Nortriptyline	1.32	2.14	11.62	120.43	1.44	1.26
	Tioridazine	2.06	1.28	1.62	1.26	0.36	0.37
18	Clomipramine	2.86	0.38	19.70	3.90	5.40	8.73

[b]Positive analysis for opiates, cocaine and phenobarbital.
– Sample not available.
FL Feeding larvae.
PFL Post-feeding larvae.

in blood samples, while in post-feeding maggots from the same samples, there was a clear decrease from that observed in the feeding specimens (cases 1, 4,10, 11). This is due to the maggots storing ingested food materials in their crop, located at the anterior end of the digestive system. This structure expands greatly during the feeding portion of the life cycle, but empties very quickly following the feeding period (Greenberg 1991) and gut contents are rapidly digested. During the post-feeding stage, the rate of drug elimination exceeds the rate of absorption, thus causing a decrease in drug concentration in the maggot.

The distribution of cocaine shows a slightly different pattern (Table 2). Cocaine was detected in 8 out of the 18 cases and was combined with opiates in all cases. As for opiates, concentrations of cocaine in liver samples were significantly higher than for blood samples and post-feeding maggots. In 4 of the cases (cases 2, 3, 4 and 10) concentrations of cocaine in feeding maggots were similar to those observed in liver samples. This is consistent with the stability of the drug which is not always homogeneous. Usually cocaine levels in blood decrease rapidly following death, hydrolyzing into ecgonine methyl ester (Isenschmid et al. 1989). Cocaine appears more concentrated in liver tissues and actively feeding maggots in which levels of cocaine detected have been significantly higher than in blood samples, with the exception of 3 cases (cases 6, 10, 14). While there were 3 cases (3, 4, 9) in which the cocaine concentrations were higher than for the corresponding blood samples, the general pattern was for post-feeding maggots to have a lower concentration than blood samples and feeding maggots, consistent with metabolism and elimination of the drug.

Antidepressants (Table 3), as well as levomepromazine, amitriptyline and nortriptyline (cases 16, 17), were more concentrated in feeding maggots than in the corresponding blood samples, but lower than in the liver samples. Only clomipramine (case 18) was detected at higher levels in both feeding and post-feeding maggots than found in blood and liver tissue samples, suggesting a greater affinity between blowfly maggots and phenotiazines or problems in metabolism and/or elimination. Compared with narcotics, post-feeding maggots associated with antidepressants demonstrated a high concentration of these drugs in their tissues. For example, concentrations of phenobarbital in cases 6 and 15 were higher for post-feeding maggots than for the younger, actively feeding maggots, consistent with a bioaccumulation. This may be attributed to its basic molecular structure (pyrimidine ring) that is extremely lipophilic and easily retained in adipose tissues of the maggots (Sadler et al. 1997c). By contrast, phenobarbital concentrations in feeding and post-feeding maggots were significantly lower than those for associated blood and liver samples.

Except for the antidepressants, all drug levels detected in post-feeding maggots were significantly lower than for actively feeding maggots. This is also consistent with the results illustrated by Pien et al. (2004) who recently detected nordiazepam and its metabolite oxazepam in blowfly larvae and puparia with levels of benzodiazepine declined through days 5 and 6 suggesting its metabolization and possible bioaccumulation rather than excretion. Our results suggest that for opiates and cocaine, fully developed and

actively feeding maggots will provide the best samples for toxicological analyses. However, as some phenotiazines, such as levomepromazine and clomipramine, as well as barbiturates such as phenobarbital, significantly accumulate during larval development, it must be kept in mind that the chemical properties of the drugs can greatly affect their detection in maggots.

Conclusions

The potential use of insects as alternative samples for detection of drugs and toxins has been well documented in the literature, however there are some limitations. These include the bioaccumulation of drugs throughout the larval development and the potential correlation between drug concentrations in maggots and the tissues used as a food source (Pounder 1991). The few cases presented in this study cannot be considered a representative sample and our results provide only an indication of the potential value of entomological evidence. However, these results do suggest a predictable pattern of drug distribution, consistent with the chemical properties of the drugs and stage of larval development. Due to the high sensitivity of the analytical techniques used in this study, drugs were detected in all human tissues, feeding maggots and post-feeding maggots, both reared and directly collected from the corpse.

Maggots, even if only as qualitative specimens, can be considered as reliable samples for detection of drugs, supporting the final diagnosis of narcotic intoxication. This is particularly true for bodies in advanced stages of decomposition where poisoning is suspected. In skeletonized remains where no human soft tissues are present or they have decomposed to the point where drugs cannot be detected due to high levels of decomposition, insects can prove to be a more suitable specimen for analyses, with less decomposition interference (Kintz et al. 1990a; Nolte et al. 1992). Products of tissue decomposition can alter the final quantitative determinations of drugs in body fluids and solid organs, including skeletal muscle. Some comparative analyses of toxicological results have shown greater sensitivity using Diptera larvae instead of putrefied cadaver tissues (Nolte et al. 1992; Kintz et al. 1990c). In our study, among the 18 drug-related deaths only 1 body (case 15) was partially skeletonized with very little soft tissue still present due to the action of local macrofauna and Diptera larvae; the results of toxicological analysis performed on the available tissues and on Diptera larvae collected directly from the corpse (as illustrated in Table 3) are consistent with the expected pattern of drug accumulation for phenobarbital. Some other studies have demonstrated that drugs could be detected in maggots but not in associated soft tissues (Kintz et al. 1990a; Levine et al. 2000; Williams and Pounder 1997) but it is also true that the absence of a drug from feeding larvae does not necessarily imply its absence from the food source (Sadler et al. 1997c).

Several workers remain skeptical of the potential value of entomotoxicology in forensic investigations, regarding this as a laboratory curiosity (Gaudry et al. 2001) or, at worst, a scientific imposter (Tracqui et al. 2004). We strongly believe that as suggested by Levine et al. (2000), "all reasonable steps must be undertaken to perform as comprehensive a drug screen as possible" in bodies where there is a strong indication that the death may be drug-related. This includes use of any available human tissues as well and entomological specimens. As for any technique when using entomological samples, factors affecting toxicological analyses of entomological materials and the limitations to their interpretation must be kept in mind.

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