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Barbiturates and Analgesics in *Calliphora vicina* Larvae

REFERENCE: Sadler DW, Robertson L, Brown G, Fuke C, Pounder DJ. Barbiturates and analgesics in *Calliphora vicina* larvae. J Forensic Sci 1997;42(3):481–485.

ABSTRACT: *Calliphora vicina* larvae were reared on artificial foodstuffs spiked with a range of concentrations of aspirin (acetylsalicylic acid), sodium salicylate, paracetamol, sodium aminohippurate, amphetamine sulfate, and the barbiturates thiopentone, phenobarbitone, amylobarbitone, barbitone, and brallobarbitone. Larvae were harvested at either day 6, 7, or 8 for analysis of drug content. Paracetamol, aspirin, amylobarbitone, and thiopentone were not detected in larvae fed on foodstuff containing drug concentrations equivalent to those expected in skeletal muscle from fatal human overdoses. Drug concentrations in larvae (expressed as larva: Foodstuff drug ratio) were 12–14% for phenobarbitone, 9–11% for sodium salicylate, 10% for aminohippurate, 18–19% for brallobarbitone, 41% for amphetamine, and 50–67% for barbitone. Amylobarbitone became detectable in larvae fed on higher drug concentrations (larva: Foodstuff drug ratio 2.6–21%). Higher levels of thiopentone and phenobarbitone were lethal to the larvae. Despite sharing similar basic structures, related drugs were each handled very differently by the larvae. We found it impossible to predict, on the basis of chemical structure, which drugs are likely to be detectable in *Calliphora vicina* larvae and in what ratio to the drug-spiked foodstuff. Drug concentrations in larvae are significantly lower than in their food source and the absence of a drug from feeding larvae does not necessarily imply its absence from the food source.

KEYWORDS: forensic science, forensic toxicology, forensic entomology, entomotoxicology, calliphora, larvae, salicylates, paracetamol, aminohippurate, amphetamine, barbiturates

The use of fly larvae as alternative toxicological specimens, first reported by Beyer in 1980 (1), has become an established approach in which conventional specimens are either not available or unsuitable for analysis (2,3). Despite the promise of this novel approach, there have been few systematic experimental studies into the metabolism of common drugs by fly larvae (4–6). We fed *Calliphora vicina* larvae on an artificial foodstuff spiked with different concentrations of several common drugs. Target drug concentrations included levels equivalent to those expected in skeletal muscle from a case of human fatal poisoning.

Materials and Methods

Calliphora vicina larvae were reared on an artificial foodstuff, made by combining muscle, powdered egg and agar, spiked with various concentrations of acetylsalicylic acid (aspirin), sodium salicylate, paracetamol (acetaminophen), aminohippuric acid, amphetamine sulfate, sodium amylobarbitone, sodium phenobarbitone, sodium thiopentone, sodium barbitone, and sodium brallobarbitone. Larvae were harvested for analysis at either day 6, 7, or 8 and analyzed for drug content.

Preparation of Artificial Foodstuff

500 g of fresh lean drug-free lower limb muscle was cut into small pieces and homogenized with 100-mL distilled water in a blender for 40 s (Waring 8011, Commercial, USA). The desired amount of drug and 30-g powdered whole egg, each dissolved in 100-mL distilled water, were added. The mixture was homogenized to the consistency of a thick milk shake and placed in an oven at 55°C for 1 h. A 45-g quantity of powdered agar (Sigma agar-agar) dissolved in 1 L of boiling distilled water was stirred vigorously for 2 min until it thickened. The agar solution was thoroughly stirred into the warmed muscle mixture and transferred into a 40 by 20 by 5 cm steel tray. After cooling, the foodstuff was cut into a hundred 3 by 2 by 1 cm portions and stored at −20°C. From each batch of foodstuff five samples, one from each corner of the tray and one from the center, were analyzed to assess homogeneity. During foodstuff preparation, correction was made for drug loss due to heat degradation, which was assessed for each drug.

Rearing of Larvae

Fresh bluebottle (*Calliphora vicina*) eggs were transferred onto thawed portions of drug-spiked foodstuff. Larvae typically emerged from the eggs and commenced feeding within 12–24 h. The day of emergence was designated day 1. Larvae were reared in a Gallenkamp incubator at 20–24°C, 70–90% humidity and with cyclical artificial lighting simulating 16 h daylight and 8 h darkness. Further fresh portions of foodstuff were introduced as required. Triplicate rearings of larvae at days 6, 7, and 8 were harvested, washed copiously in cold tap water and dried on absorbent material. Larvae were killed by freezing to −20°C and stored prior to analysis. Foodstuff and excreta remaining after harvesting the larvae in the second series of experiments was also collected for analysis.

Analytical Techniques

All methodologies used in this study have been developed in our laboratory and are used routinely for the analysis of drugs in various biological matrices. Appropriate standards were extracted concurrently with each individual series of drug extractions. All quantitations were performed using an internal standard. Calibration curves and standards were prepared for each drug studied, using a range of concentrations ($n = 6$) of the appropriate drug

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Received 28 Dec. 1995; and in revised form 5 Aug. 1996; accepted 12 Sept. 1996.

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spiked into drug-free larvae or foodstuff. All calibration curves had a correlation coefficient of >0.99. All samples were extracted and analyzed in duplicate and the values were only accepted if agreement was within 5% of the mean. The limits of detection for the drugs are sub-therapeutic.

In the first series of experiments larvae were reared on artificial foodstuffs containing 1000 mg/kg acetylsalicylic acid, 250 mg/kg paracetamol, 100 mg/kg amylobarbitone, and 100 mg/kg phenobarbitone. The larvae were harvested for analysis on day 8.

Samples of artificial foodstuff were first homogenized with the blender. A 5-g sample of homogenate was weighed out accurately, made up to 20 mL with distilled water and homogenized further (Ultra Turrax T25, IKA, Germany). Five gram samples of larvae were weighed out accurately and counted. The samples were first homogenized with 5-mL distilled water, made up to 20 mL with distilled water and then homogenized further.

The method of extraction for acetylsalicylic acid and paracetamol was similar to that used in previously published studies (4,7). A 400-μL sample of larval or foodstuff homogenate was vortex mixed with 100 μL 0.05 M sulfuric acid and 50 μL of internal standard. The internal standard was, for acetylsalicylic acid, 0.5 mg/mL 2-acetoamidophenol in methanol and for paracetamol, 5 mg/mL 2-acetoamidophenol in methanol. The sample was transferred to a non-preconditioned Chem Elut™ column (1219-8002, Varian Sample Preparation Products, P.O. Box 234, Cambridge, CB1 1PE, UK), according to the manufacturer's instructions. The sample was extracted under vacuum using a Vac-elut system and allowed to stand for 5 min. The drugs were eluted with 4 mL of chloroform: iso-propanol (1:1). The eluate was evaporated to dryness on a heating block under a stream of dry air at 55°C. The extract was resuspended in 1 mL methanol and 20 μL was injected onto a high performance liquid chromatography system (HPLC): instrument, Perkin-Elmer isocratic LC pump 250 with Perkin-Elmer spectrophotometric detector LC 90 UV and Waters autoinjector 712 WISP; column, Apex II ODS 5 μm, 150 by 2.6 mm internal diameter with a 20 mm guard column. For acetylsalicylic acid the mobile phase was 400-mL distilled water, 75-mL acetonitrile, and 25-mL acetic acid; flow rate, 1 mL/min; UV detector at 280 nm. For paracetamol the mobile phase was 425-mL distilled water, 50 mL acetonitrile and 25 mL glacial acetic acid; flow rate, 1 mL/min; UV detector at 255 nm.

The method of extraction for the barbiturates was similar to a previously published method (7). A 400-μL sample of larval or foodstuff homogenate was vortex mixed with 200-μL internal standard (10 mg/mL secobarbitone sodium solution) and 400-μL 0.5 M sodium dihydrogen orthophosphate buffer, pH 9. A 600-μL sample was transferred to a non-preconditioned Chem Elut column and extracted under vacuum using a Vac-elut system and allowed to stand for 5 min. The drugs were eluted with 4 mL of dichloromethane, evaporated to dryness and reconstituted in 1-mL methanol. For the barbiturates the HPLC mobile phase was 110 mL methanol with 135 mL acetonitrile and 200 mL 0.14 M sodium dihydrogen orthophosphate, made up to 500 mL with distilled water; flow rate 1 mL/min; UV detector at 205 nm.

In a second series of experiments, larvae were reared on artificial foodstuffs containing 2000 mg/kg paracetamol, 2000 and 4000 mg/kg sodium salicylate, 2000 mg/kg sodium aminohippurate, and 50 mg/kg amphetamine sulfate. The larvae were harvested for analysis on day 6.

The procedure for homogenization of foodstuff and larval samples and for extraction of paracetamol were as described above for paracetamol in the first series of experiments. The internal standard solution used was 2-acetoamidophenol at 5 mg/mL for artificial foodstuff and 0.5 mg/mL for larvae. The extract of foodstuff and larvae were resuspended in 1 mL and 100 μL of methanol, respectively.

For extraction of sodium salicylate, 1 g of artificial foodstuff was weighed out accurately and homogenized with 1 mL of 5 mg/mL 2-acetamidophenol internal standard solution and 8 mL of distilled water. A 1-g sample of larvae was counted and homogenized with 10 μL of internal standard solution and 3-mL distilled water. Foodstuff and larval homogenates were then centrifuged at 3000 rpm for 5 min. A 100-μL sample of foodstuff supernatant was diluted ten times with distilled water and 20 μL injected directly onto the HPLC. A 400-μL volume of larval homogenate supernatant was vortex mixed with 100 μL of 0.05 M sulfuric acid. The sample was vacuum extracted as for paracetamol and resuspended in 100 μL of methanol. A 20-μL sample was injected onto the HPLC; the mobile phase was 800-mL distilled water, 200-mL acetonitrile, and 50-mL acetic acid; flow rate 1 mL/min.; UV detector at 300 nm.

For extraction of sodium aminohippurate, 1 g of artificial foodstuff was weighed out accurately and homogenized with 1 mL of 5 mg/mL paracetamol internal standard solution and 8 mL of distilled water. To 1-mL of homogenate were added 9 mL of methanol. A 2-g sample of larvae was counted and homogenized with 100 μL of 5 mg/mL paracetamol internal standard solution and 3.9-mL methanol. The foodstuff/methanol mixture and larval homogenate were then centrifuged at 3000 rpm for 5 min. A 20-μL sample of supernatant was injected onto the HPLC; the mobile phase was 470-mL distilled water, 15-mL acetonitrile, and 15-mL acetic acid; flow rate 1 mL/min; UV detector at 280 nm.

For extraction of amphetamine sulfate, 2 g of artificial foodstuff was weighed out accurately and homogenized with 200 μL of 500 μg/mL mephentermine internal standard solution and 5.8 mL of distilled water. After addition of 1 mL of 0.05 M sulfuric acid, 2 mL of foodstuff homogenate was rotator mixed with 3 mL of ethyl acetate for 15 min (Spiramix 10, Deney, U.K.). After centrifugation the ethyl acetate layer was aspirated and discarded. A further 3-mL heptane was added and the procedure repeated. To the aqueous layer were added 1 mL of sodium hydroxide and 3 mL of ethyl acetate, and the mixture was again rotator mixed and centrifuged. The organic layer was retained and, after addition of 1 drop of acetic acid, evaporated to dryness at 55°C. The residue was resuspended in 100-μL of methanol. A 20-μL sample was injected onto the HPLC system. The HPLC method was similar to a previously published method (8). The mobile phase was 425-mL distilled water containing 9.8 g phosphoric acid and 5.2 g triethylamine, and 75-mL acetonitrile, adjusted to pH 3.0 with 2 N sodium hydroxide; flow rate 1 mL/min.; UV detector at 210 nm.

In a third series of experiments larvae were reared on foodstuffs containing 0.5, 1 & 2 mmol/kg of thiopentone, amylobarbitone, and 0.5 & 1 mmol/kg phenobarbitone, barbitone and brallobarbitone. Larvae were harvested for analysis on day 7. The extraction and analysis of barbiturates in both foodstuff and larvae were as described above for amylobarbitone and phenobarbitone in the first series of experiments.

Using the above methods, the limits of detection (expressed per L of larval homogenate) were: for acetylsalicylic acid, 0.05 mg/L; sodium salicylate, 0.05 mg/L; paracetamol, 0.05 mg/L; sodium aminohippurate, 1 mg/L; amphetamine sulfate, 0.02 mg/L; phenobarbitone, 0.1 mg/L; amylobarbitone, 0.5 mg/L; barbitone, 1 mg/L; thiopentone, 0.8 mg/L; brallobarbitone, 1 mg/L. The limits of detection for these drugs are sub-therapeutic.

Results

Table 1 shows the drug concentrations in day 8 larvae reared on low levels of phenobarbitone, amylobarbitone, paracetamol and acetylsalicylic acid and in day 6 larvae reared on aminohippurate, amphetamine, and higher levels of sodium salicylate and paracetamol. Table 2 shows the drug concentrations in day 7 larvae reared on various concentrations of thiopentone, phenobarbitone, amylobarbitone, barbitone and brallobarbitone. For convenience, larval drug concentrations are also expressed as a percentage of the drug concentration present in the foodstuff, these percentages being derived from data presented in the same tables.

Discussion

In the first series of experiments, target drug concentrations in the artificial foodstuff were equivalent to those expected in skeletal muscle in a case of fatal human poisoning. If we accept that drug levels in skeletal muscle (expressed in mg/kg or μg/g) are broadly similar to levels in blood (expressed in mg/L or μg/mL) (9), then artificial foodstuffs containing 250 mg/kg paracetamol, 1000 mg/kg acetylsalicylic acid, 100 mg/kg amylobarbitone, and 100 mg/kg phenobarbitone represent equivalent blood levels within the published human fatal ranges of 160–387 mg/L for paracetamol, 61–7320 mg/L for acetylsalicylic acid, 13–96 mg/L for amylobarbitone, and 10–300 mg/L for phenobarbitone (10) (Table 1). Of these four drugs, only phenobarbitone was detected in day 8 larvae. That paracetamol was not detected confirms earlier findings that the drug is efficiently eliminated by *Calliphora vicina* larvae (4).

In the second group of experiments, the effects on larvae of greatly increasing the target concentrations of paracetamol and salicylate in the foodstuff were explored. Paracetamol became detectable in day 6 larvae reared on foodstuff containing 2000 mg/kg. However, the low levels of drug detected in larvae (larva:food ratio of 0.2%) likely reflects the mere presence of drug-laden food in the crop of larvae which are still actively feeding on day 6.

Sodium salicylate was substituted for acetylsalicylic acid in the preparation of artificial foodstuff due to its greater water solubility, enabling higher drug concentrations to be achieved. At these higher levels of 2000 and 4000 mg/kg salicylate became detectable in larvae in significant amounts (larval:food ratios of 8.6% and 10.7%). For comparative purposes, larvae were also fed on aminohippurate, a marker of excretory function in humans. Aminohippurate was detectable in comparable levels to sodium salicylate in day 6 larvae (larval:food ratio of 9.7%). Amphetamine, a common drug of abuse, was detectable at still higher levels in day 6 larvae (larval:food ratio of 41%, foodstuff concentration 45 mg/kg). Paracetamol, acetylsalicylic acid, aminohippurate, and amphetamine all have a similar basic benzene ring structure but differ in the number, position, and nature of their side chains. Paracetamol, acetylsalicylic acid, and aminohippurate each have two relatively simple side chains, whereas amphetamine has a single, longer, branched side chain. The molecular weights of these four drugs are broadly similar: paracetamol, 151.2; acetylsalicylic acid, 180.2; aminohippurate, 194.2; amphetamine, 135.2. The four drugs differ more widely in their other physical properties (aspirin soluble 1 in 300 water, pKa 3.5; paracetamol soluble 1 in 70 water, pKa 9.5; aminohippurate soluble 1 in 45 water, pKa 3.6; and amphetamine sulfate soluble 1 in 9 water, pKa 9.9). The reason why amphetamine appears to accumulate in larvae to a greater degree than the other three drugs is not apparent.

In the third series of experiments, the way in which larvae handle barbiturates as a group was studied. To facilitate comparisons,

TABLE 1—*Larval concentrations of phenobarbitone, amylobarbitone, paracetamol, acetylsalicylic acid, aminohippurate, and amphetamine.*

Drug	Drug Conc. in Foodstuff (mg/kg)		Harvest Day	Mean Drug Conc. in Food Residue (mg/kg) (Range), n = 3	Mean Larval Weight (mg)	Mean Drug Conc. in Larvae (mg/kg) (Range), n = 3	Larva: Food Drug Ratio as %
	Target	Actual Mean (Range), n = 5					
Series 1							
Phenobarbitone	100	58.4 (51.8– 62.9)	8	n.a.	20	7.2 (4.1– 12.4)	12.3
Amylobarbitone	100	106.8 (102– 113)	8	n.a.	34	n.d.	0
Paracetamol	250	241 (222– 258)	8	n.a.	31	n.d.	0
Acetylsalicylic acid	1000	800 (726– 982)	8	n.a.	39	n.d.	0
Series 2							
Na Salicylate	2000	1870 (1620–2200)	6	2760 (1850–4540)	60	160 (40 –285)	8.6
Na Salicylate	4000	3100 (2450–3430)	6	2680 (2200–2970)	51	331 (61.8–683)	10.7
Paracetamol	2000	2290 (2000–2720)	6	3130 (2000–4610)	48	5.6 (4.9– 6.4)	0.2
Aminohippurate	2000	1520 (1390–1620)	6	2170 (1530–2730)	65	147 (71.6–236)	9.7
Amphetamine	50	44.8 (43.3– 45.9)	6	36.3 (25.3– 52.6)	70	18.5 (11.3– 22.5)	41

NOTE:—n.a. = not available; n.d. = not detected;

TABLE 2—*Larval concentrations of thiopentone, phenobarbitone, amylobarbitone, barbitone, and brallobarbitone.*

Mean Drug Conc. in Foodstuff (mmol/kg)							
Drug	Target	Actual Mean (Range), n = 3	Harvest Day	Mean Larval Weight (mg)	Drug Conc. in Larvae (μmol/kg) Mean (Range), n = 3	Larva: Food Drug Ratio as %	Notes
Series 3							
Thiopentone	0.5	0.53 (0.48–0.61)	7	30	0	0	
Thiopentone	1	1.35 (1.03–1.64)	7	—	—	—	All larvae died
Thiopentone	2	2.43 (2.33–2.64)	7	—	—	—	All larvae died
Phenobarbitone	0.5	0.62 (0.44–0.71)	7	93	87.3 (82– 94)	14	Very large larvae
Phenobarbitone	1	1.42 (0.99–2.04)	7	—	—	—	All larvae died
Amylobarbitone	0.5	0.81 (0.67–0.95)	7	100	0	0	Very large larvae
Amylobarbitone	1	1.25 (1.2 –1.36)	7	63	32.2 (31– 34)	2.6	
Amylobarbitone	2	2.39 (2.16–2.583)	7	19	498 (442–514)	21	Variable size larvae
Barbitone	0.5	0.44 (0.22–0.61)	7	58	296 (233–394)	67	
Barbitone	1	1.28 (1.17–1.43)	7	33	634 (658–670)	50	Many larvae dead
Brallobarbitone	0.5	0.537 (0.48–0.57)	7	41	94 (73–115)	18	Many larvae dead
Brallobarbitone	1	0.982 (0.88–1.13)	7	10	186 (142–268)	19	Many larvae dead

foodstuffs were prepared containing equimolar concentrations of barbiturates and levels in larvae were expressed in mmol/kg larvae rather than in mg/kg. In these terms, molar concentrations of 0.5, 1 and 2 mmol/kg foodstuff represent respective equivalent blood levels of 116, 232, and 464 mg/L for phenobarbitone; 113, 226, and 452 mg/L for amylobarbitone; 92, 184, and 368 mg/L for barbitone; 121, 242, and 484 mg/L for thiopentone; and 144, 287, and 574 mg/L for brallobarbitone. Human fatal blood levels are 10–300 mg/L (mean 86 mg/L) for phenobarbitone; 13–96 mg/L for amylobarbitone; 70–170 mg/L for barbitone, 6–392 mg/L for thiopentone; and 10–25 mg/L for brallobarbitone (10). Although this group of drugs share the same basic pyrimidine ring structure, they differ in their side chain structure. This clearly has a major impact on the way in which different barbiturates are metabolized by *Calliphora vicina* larvae. Specifically, barbitone, the oxybarbiturate with the simplest chemical structure, accumulates significantly in larvae (larval:food drug ratio of 50–67%) and amylobarbitone, an oxybarbiturate with a long, branched side chain, appears to show dose-dependent accumulation in larvae (larval:food drug ratio of 2.6–21%). In contrast, thiopentone, a thiobarbiturate with a long, branched side chain, and phenobarbitone, an oxybarbiturate with a benzene ring as its side group, are both apparently lethal to larvae at equimolar concentrations. Thiopentone was not detectable in larvae and phenobarbitone in only low relative concentrations (larval:food drug ratio of 14%). Marked variability in larval size and mortality were also noticeable amongst the larvae fed on the various barbiturates (Table 2). Other drugs have been shown to have a profound effect on the rate of larval development in several fly species. Larval development is accelerated by the presence in the food source of morphine, cocaine, or methamphetamine (11–13). The duration of the post-feeding period is prolonged by the presence of amitriptyline and shortened by phencyclidine (3,14).

That larvae in each of the three series of experiments were harvested on different days is unlikely to cause comparative difficulties as previous research has shown that concentrations of most drugs are at their highest in larvae between days 6 and 8 of the life-cycle (5). In contrast, larval drug concentrations fall precipitously in larvae which have ceased feeding and are preparing to pupate (6).

Our results overall indicate that it is impossible to predict on the basis of chemical structure which drugs are likely to be detectable in *Calliphora vicina* larvae. The same is likely to hold for other fly species. Consequently, the absence of a drug in feeding larvae may not necessarily imply absence of drug in the corpse. Many of the drugs studied were not detectable in *Calliphora vicina* larvae, using the standard methods used in our laboratory. These methods are used routinely to quantify drug concentrations in biological fluids and tissues, at levels ranging from sub-therapeutic to fatal. Clearly, use of more rigorous drug extraction techniques, such as those used on hair samples, may give improved drug recovery and detection. This has been our experience in the analysis of amitriptyline in the larvae and pupae of *Calliphora vicina* comparing two different analytical methods (15). Where a drug is detectable in larvae, extrapolation from that concentration to the concentration in the corpse may be difficult if not impossible in the absence of test study data. As a general rule, all that can be said is that drugs, if detectable, are present in *Calliphora vicina* larvae at lower concentrations than in the foodstuff.

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