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Effects of Tramadol on the Development of *Lucilia sericata* (Diptera: Calliphoridae) and Detection of the Drug Concentration in Postmortem Rabbit Tissues and Larvae

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ABSTRACT

The present study was conducted to investigate the tramadol impact on the larval development of *Lucilia sericata*. Larvae of the green bottle blow fly *Lucilia sericata* (Meigen) were reared on rabbit carcasses treated with tramadol at concentrations; 550, 1100 and 2200 mg kg^{-1} through oral doses. These concentrations were similar to those normally encountered in cases of death due to tramadol overdoses. Tramadol was detected in studied tissues and larvae by using High Performance Liquid Chromatography (HPLC). The concentrations of tramadol in various tissues were significantly increased with the initial dosage. Concentrations of tramadol in larvae were significantly lower than those found in tissues. The maximum sizes were attained earlier in larvae reared in rabbits treated with tramadol than those reared on the control rabbit. However, the total developmental period increased significantly with increasing the initial administrated dosage. Therefore, care must be taken in fatal tramadol cases and toxicological analysis of tissues and larvae, or both should be completed before final estimating of postmortem interval (PMI).

Key words: Development, forensic toxicology, larvae, *Lucilia*, tramadol

INTRODUCTION

Entomotoxicology is a relatively new branch of forensic entomology. The potential use of necrophagous insects for detecting drugs and other toxins in decomposing carcasses have been widely demonstrated (Nolte *et al*., 1992; Goff and Lord, 2001; Campobasso *et al*., 2001; Introna *et al*., 2001; Kharbouche *et al*., 2008). The analysis of larvae found in cadavers can, therefore, contribute to the qualitative identification of drugs present in the corpse (Nolte *et al*., 1992; Kintz *et al*., 1990a, b; Introna *et al*., 1990). In addition, drugs in putrefied tissues may have an influence on the development of the necrophagous Diptera that can affect the estimation of the PMI (Goff *et al*., 1991, 1992, 1993; Bourel *et al*., 1999; Carvalho *et al*., 2001; O'Brien and Turner, 2004). Drug levels in larvae could also be correlated to drug concentrations in tissues eaten by the insects, providing valuable information to elucidate the cause of death. The larval behavior toward several substances is unsure; consequently, their use for qualitative identification and quantitative analysis of drugs or toxins are strongly limited (Sadler *et al*., 1997a, b; Tracqui *et al*., 2004).

The green bottle blow fly *Lucilia sericata* is widespread throughout the major zoogeographical regions (Greenberg and Povolny, 1971; Smith, 1986) and is one of the dominant flies of forensic

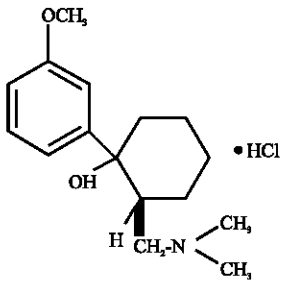

Fig. 1: Molecular structure of tramadol

importance in Egypt (Tantawi *et al*., 1996). It is one of the earliest arriving fly species on remains, with oviposition occurring typically only few hours after death (Byrd and Castner, 2001). Currently, this species is gaining a new-found importance in the field of forensic entomology as one of the primary species utilized to indicate the PMI in human deaths (Vanin *et al*., 2008). Also, larvae of this species are most commonly encountered in cases of human myiasis (Chigusa *et al*., 1998; Hira *et al*., 2004) and are extensively used for maggot therapy as it has a propensity to invade healthy tissues (Young, 1997; Sherman *et al*., 2000; Wayman *et al*., 2000).

Over the past two decades, the increase in antidepressant drug-related deaths (tramadol, amitriptyline) justifies the great interest arisen by this discipline in forensic medicine (Goff *et al*., 1993; Michaud *et al*., 1999; Musshoff and Madea, 2001; Loughrey *et al*., 2003). Tramadol is a centrally acting analgesic, used for the treatment of moderate to severe pains (Fig. 1) and has become the most prescribed opioid worldwide (Shipton, 2000). Tramadol in experimental and clinical trials exhibited a good analgesic efficacy and potency comparable to codeine (Lehmann, 1994; Grond *et al*., 1995). In acute postoperative pain its efficacy was similar to that of morphine. In addition, a long-term tramadol treatment in patients with nocigenic, neurogenic, or sympathogenic pain was as effective as sustained release of morphine and significantly better than either amitriptyline or carbamazepine (Vickers and Paravicini, 1995).

Tramadol has a good tissue affinity and the ability to cross the blood-brain barrier and placental barrier (Gronod and Sablotzki, 2004). This drug has been found to produce numerous positive responses in vertebrates, including analgesia for moderate to severe pain; antitussive, (Kukanich and Papich, 2004) antidepressant, (Faron-Gorecka *et al*., 2004), immunostimulatory effects (Gronod and Sablotzki, 2004) and an ability to lower glucose in diabetics (Cheng *et al*., 2001). Forensic autopsies have been shown to constitute a unique source for the identification of drug-related deaths (Jonsson *et al*., 2004). Using forensic materials, Clarkson *et al*. (2004) reported that tramadol might be a significant contributor to lethal intoxication when taken in excess with other drugs that depress central nervous functions, such as analgesics, muscle relaxants and antidepressants.

This study deals with the effect of tramadol on the rate and pattern of development of *L. sericata*. It also concerns, the detection of tramadol in the viscera of the animal model. Correlations between drug concentrations in the rabbits' tissues and its concentrations in the larvae fed on those tissues were also investigated.

MATERIALS AND METHODS

Administration of tramadol into rabbits: Experiments were carried out in September 2009 at Moharrem Bey District, Alexandria, Egypt. Four healthy female rabbits (*Orctylagus cuniculus*),

each weighing approximately 2 kg were used for this study. Three rabbits received tramadol (as tramadol hydrochloride solution) as a single oral dose of 550 (R1), 1100 (R2), 2200 (R3) mg kg^{-1} body weight (Matthiesen *et al*., 1998). One rabbit (R0) which was used as a control animal (treated with saline), was run interspersed concurrently with the drug-treated animals. The rabbits were immediately killed by strangulation and dissected.

Sampling of tissues for toxicological analysis

Blood sampling: By a sterile scalpel, an incision was made in the abdomen of each rabbit and samples of 15 mL were collected into heparinised vacutainers from the cardiac artery after tramadol administration. The blood samples were immediately placed on ice, centrifuged at 2000x g at room temperature for 5 min, the separated plasma was then stored at -80°C until analysis to determine the tramadol concentration.

Tissue preparation: Samples of brain, kidney, liver, bile, lung, fat, gastric content and abdominal muscles were taken. 1.0 g of each solid tissue was dissected out over a stainless steel plate cooled with ice. After weighting, the solid tissue were rinsed in phosphate buffer, wrapped in a piece of aluminum foil, sprayed with ethylene chloride and immediately frozen at -20°C till analysis. Three samples per tissue per carcass were taken. After sampling, rabbits were sutured to reconstitute their initial anatomy.

Sampling of larvae for toxicological analysis: Laboratory colonies of *L. sericata* were established from third feeding instars collected from exposed rabbit carcasses at Moharrem Bey District, Alexandria, Egypt. Gravid females were allowed to oviposit on minced meat and eggs were collected within 30 min of oviposition. Each rabbit carcass was seeded in the natural openings by approximately 500 eggs of *L. sericata*. Carcasses were placed into plastic boxes with wire netting. These boxes were held in a constant room temperature of 25°C.

At regular intervals 6-12 h, larvae were randomly sampled from each rabbit, killed immediately in boiling water (Adams and Hall, 2003) and their length measured. During the feeding larval phase, 20 of 3rd feeding instar larvae were taken for toxicological analysis (Gagliano-Candela and Aventaggiato, 2001). The rest were allowed to complete their development. Pupariation and adult ecolsion were investigated at 6 h intervals.

Toxicological analysis

Sample extraction: Solid tissue were homogenized for 30 sec and diluted with 1 mL of water. Extraction was accomplished with liquid-liquid extraction. Briefly, in 10 mL tube, 0.5 of serum or 1.0 mL of tissue suspension was alkalinized with 500 µL of sodium hydroxide 0.5 mol L^{-1}. Samples were extracted with 5 mL of ethyl acetate and centrifuged at 2000xg for 10 min. The organic layer was evaporated under a gentle stream of nitrogen. The residue was redissolved in 1 mL methanol and an aliquot 10 µL was used for injection.

Sample analysis: Tramadol concentrations were analyzed by high performance liquid chromatography (HPLC Agilent 1100 series) with UV-Visible spectrophotometric detector at 280 nm. Separation was achieved using prevail C18, 5µ, 250×4.6 mm column, the mobile phase used was Methanol: Water (80: 20) containing triethyl amine and the flow rate of the mobile phase

was 1.0 m min^{-1}, tramadol retention time was 11.16 min (tr: 11.16). The extraction recovery was estimated by comparing the slope of the standard curves of extracted standards with that for unextracted standards. The recovery was 82%.

Chemicals and reagents: Tramadol was supplied by Grunenthal (achen, Germany). Methanol (HPLC-grade) was purchased from sigma-Aldrich chemie GmbH. D-89555 Steinheim, Germany. Sodium hydroxide was purchased from Riedel-deHaen sigma-Aldrich Laborchemikalien GmbH. D-30926 Seelze, Germany. Other reagents from different chemical sources, were analytical or HPLC grade.

Statistical analysis: Statistical analysis was performed using the program SPSS (Norusis, 2005). Data were analyzed statistically by using one-way Analysis of Variance (ANOVA) (Sokal and Rohlf, 1981) to determine the effect of tramadol dosage on the larval development. A linear regression was used to evaluate the relationship between the concentration of tramadol in the tissues and larvae against the initial dosage.

RESULTS

All blood and tissue samples from the rabbits receiving dosages of tramadol were positive for the drug. While, all samples from the control rabbit R0 were negative (Table 1). For all tissues, tramadol concentrations for rabbit R2 were 1.5 times higher than those for rabbit R1, whereas concentrations for rabbit R3 were 1.2 times higher than those for rabbit R2. The highest tramadol concentrations were detected in bile and the gastric content of rabbit R3 recording 98.4 and 124 mg kg^{-1}, respectively.

For the lethal and double lethal doses, tissues could be in the following arrangement according to their tramadol concentrations; Gastric content> bile>brain> kidney>lung>fat> liver> blood>muscles.

The concentrations of tramadol in various organs significantly increased with the initial dosage (Fig. 2). The highest correlation ($r = 1$; $p<0.01$) was recorded for liver.

Toxicological analysis showed that tramadol transferred from administered animals to the feeding larvae of *L. sericata* (Fig. 3a, b).

Table 1: Tramadol distribution (mg kg^{-1}) in different tissues and in the 3rd feeding larval instar of *L. sericata*

	Tramadol concentration				
Tissues	R0	R1	R2	R3	r[a]
Blood	0.0	23.6	39.4	54.8	0.961
Brain	0.0	58.6	84.3	97.0	0.976
Kidney	0.0	43.2	67.6	82.8	0.996
Liver	0.0	27.4	42.8	72.3	0.996
Lung	0.0	36.1	59.5	77.5	0.987
Bile	0.0	54.6	90.4	98.4	0.905
Gastric content	0.0	63.2	105	124	0.955
Fat	0.0	18.4	47.2	55.8	0.925
Muscle	0.0	20.3	34.0	48.7	0.998
Larvae	0.0	2.30	3.70	5.60	

[a]r is the correlation coefficient between larval and organ concentrations

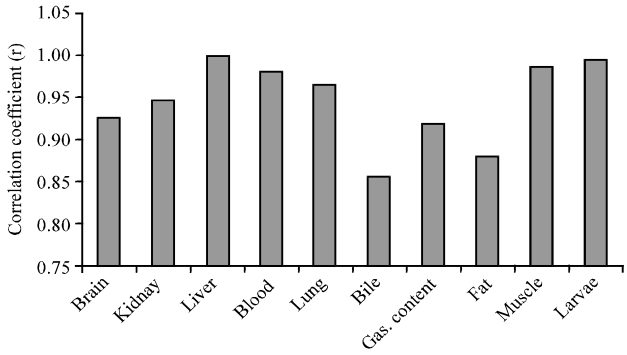

Fig. 2: Correlation coefficients between initial dosage and tramadol concentration in different tissues and third feeding instar larvae of *L. sericata*

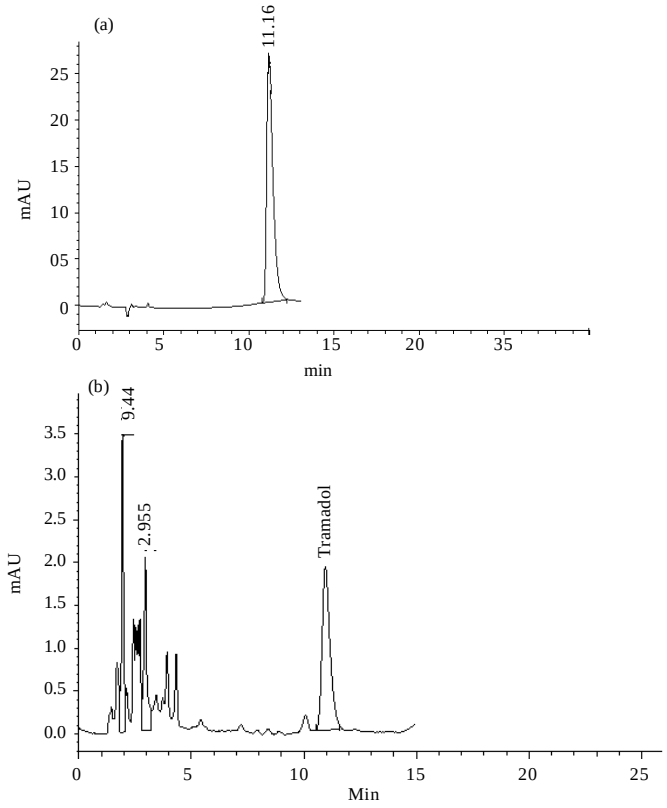

Fig. 3: HPLC chromatogram of tramadol (a) in the feeding instar of *L. sericata* and (b) (t_R: 11.16)

Concentrations of tramadol in the feeding third instar of *L. sericata* were significantly lower than those detected in the rabbit tissues. Regression analysis shows concentrations in larvae were significantly increased ($r = 0.961$, $p<0.01$) with the initial dosages of tramadol administrated as well as tramadol concentrations in rabbit organs used as a food source (in all cases $p<0.01$) (Table 1). The recorded maximal larval length was affected by the tramadol concentrations in the tissues (Fig. 4). The highest maximal larval length (14.7 mm) was recorded for larvae reared on rabbit R3. The maximum sizes were attained earlier in larvae reared in rabbits treated with tramadol than

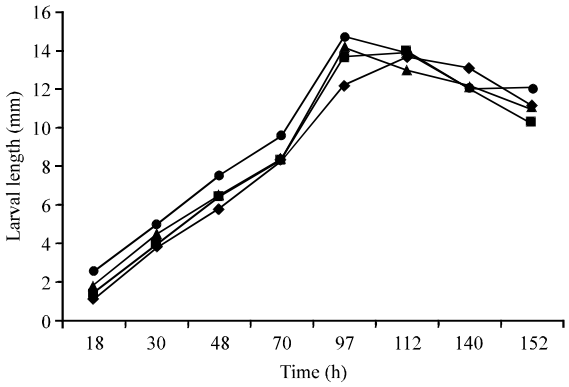

Fig. 4: Mean body lengths of L. sericata larvae (from 2nd instar to pupariation) reared on rabbit Carasses administrated different dosages of tramadol R0 = control, R1 = 550 mg kg^{-1}, R2 = 1100 mg kg^{-1}, R3 = 2200 mg kg^{-1}

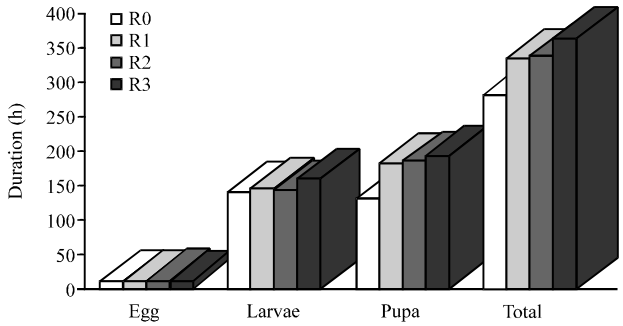

Fig. 5: Minimum duration (h) of the developmental stages of L. sericata reared of rabbit carcasses administrated different dosages of tramadol. R0 = control, R1 = 550 mg kg^{-1} R2 = 1100 mg kg^{-1}, R3 = 2200 mg kg^{-1}

those reared on the control rabbit. The mean larval length varied significantly for 30 h ($F = 86.75$; $p = 0.000$), 97 h ($F = 350$; $p = 0.000$) and 152 h ($F = 146$; $p = 0.000$). Therefore, presence of tramadol in tissues appeared to accelerate the feeding larval stages of *L. sericata*.

In this study, the presence of tramadol on rabbit tissues appeared to increase the mean minimal development times from egg to adult eclosion (Fig. 5). It ranged from 13.9 to 15.1 day for larvae reared on R1 and R3 colonies, respectively, whereas, those in R0 colony took 11.7 day only to complete their development.

Regression analysis showed significant increase in the duration of larva, pupa and total development with the initial tramadol dosage (in all cases $r = 1$; $p<0.01$).

DISCUSSION

Considering the wide use of tramadol, unintentional fatal toxicity of this drug seems to occur rarely and tramadol might hence still be a safe alternative compared to other opioids. This may be due to its low abuse potential and because less observed respiratory depression (Tjaderborn *et al.*, 2007).

As the tramadol concentration in the gastric content was the highest value, this may be useful to determine the possible time of drug administration and to distinguish oral from other routes of administration.

In present study, the higher concentration of tramadol in tissues was found in the brain, since the brain receives one-sixth of the total amount of blood leaving the heart. Some recent papers examining drugs of abuse in brain are listed (Kalasinsky *et al.*, 2000; Moriya and Hashimoto, 2003). Following the brain, kidney contains higher concentration of tramadol and this result suggests that there is a low excretion of tramadol.

Essentially, all drugs of abuse are detectable in bile. The highest concentrations of tramadol were measured in urine and bile (Musshoff and Madea, 2001). The present study supports this concept as the highest tramadol concentration was found in bile. However, in other studies, tramadol and other opioids appear to be present in higher concentrations in blood (Robertson and Drummer, 1998; Gock *et al.*, 1999).

Moreover, the recorded results showed lower tramadol concentration in the blood which is consistent with a study carried by Juzwin *et al.* (2000). Levine *et al.* (1997) analysed tramadol distribution in four postmortem cases and found that the heart blood concentrations were the lowest tramadol concentrations compared to other tissues (e.g., liver, kidney). The concentration of tramadol in the liver and kidney, in relation to blood, failed to suggest sequestration of the drug in the analysed specimens. In the present study, tramadol concentration in liver was approximately two times lower than for brain, this may be attributed to the tramadol destruction by liver. This is in agreement with Oliw and Sprecher (1991), who studied the metabolism of cis-tramaddol in human liver microsomes.

Muscles will often represent the greatest single mass of drug in a body and will therefore represent a greater body burden of drug than any other tissue mass. However, muscle is a difficult tissue to work with and care is required to ensure complete drug extraction (Williams and Pounder, 1997). Variability in the concentration of drugs has been found in muscle tissue (Langford *et al.*, 1998). This may interpret the lowest drug distribution in muscles for our results.

Larvae (maggots) found in putrefying bodies can serve as alternate samples to obtain evidence of the presence of drug in the body (Beyer *et al.*, 1980; Wilson *et al.*, 1993; Kintz *et al.*, 1994; Sadler *et al.*, 1995; Hedouin *et al.*, 1999; De-Letter *et al.*, 2000). Present study restricted to the active feeding larval instar, based on the previous studies carried by Sadler *et al.* (1995) and Hedouin *et al.* (1999, 2001). These studies found that metabolism and elimination of drugs by larvae vary considerably throughout larval development with a clear decrease in drug concentrations measured in postfeeding larvae and during pupariation. The decrease in drug concentrations suggested that only larvae actively feeding on a corpse and fully developed should be sampled for toxicological analysis because they represent the best source of drug or toxin residue.

In present study, tramadol concentration in larvae of *L. sericata* was lower than those in the tissues used as food. These results are consistent with those recorded in the larvae of *L. sericata* (Hedouin *et al.*, 1999) and the larvae of *Protophormia terraenovae* and *Calliphora vicina* (Hedouin *et al.*, 2001) reared on rabbit carcasses containing morphine. On the other hand, Introna *et al.* (1990) found that the concentrations of morphine in the larvae of *C. vicina* were quite similar to those in human liver tissues used as a food source. Other studies by Nolte *et al.* (1992) and Kintz *et al.* (1990b, 1994) found that concentrations of drugs in larvae were significantly lower than those observed in tissues.

In present study, tramadol concentrations in the larvae were significantly correlated with the initial dosage and concentrations found in tissues that are the primary food source for larvae. This is consistent with Introna *et al.* (1990), Hedouin *et al.* (1999, 2001) and Tantawi *et al.* (2001), who found a strong correlation between concentration of drugs administered and concentrations in rabbit tissues.

On the other hand, present results contradict with those obtained by Pounder (1991), Tracqui *et al.* (2004) and Strehler *et al.* (2005), who observed no correlation between drug concentrations in larvae and those in tissues on which the larvae were feeding.

Previous studies have demonstrated that presence of drugs and toxins can alter developmental rates of carrion-insects feeding on decomposed tissue from cadavers (Catts and Goff, 1992; Goff *et al.*, 1992; Bourel *et al.*, 1999; Goff and Lord, 2001; Byrd and Castner, 2001; O'Brien and Turner, 2004; Tabor *et al.*, 2004). The present study showed that presence of tramadol in rabbits tissues accelerated the larval feeding growth rate and retarded the total development. Present results were in accordance with Goff *et al.* (1991), who conducted a study for heroin effect on the development of *B. pergrina*. They found more rapid development of maggots in all treated colonies until maximum size was attained. Similar results were reported for methamphetamine (1992) and amitriptyline (1993).

Regarding to the larval growth, larvae from all colonies fed on rabbits receiving tramadol were larger and attained their maximum length earlier than those from the control colony. These results were unlike the situation with heroin and cocaine (Goff, 1993), where larvae from all colonies fed on rabbits receiving methamphetamine were smaller at maximum length (attained) earlier than those from the control colony.

Bourel *et al.* (1999) reported that if the presence of morphine in the tissues is not considered an underestimation of the PMI is possible for larvae of *L. sericata* measuring 8 to 14 mm total length. Also, Hedouin *et al.* (1999) demonstrated the potential underestimation of PMI based on the developmental analysis of necrophagous fly larvae *L. sericata* fed on the tissues of deceased rabbits previously perfused with various concentrations of morphine.

Insects may serve as reliable alternative specimens for toxicological analyses in the absence of tissues and body fluids normally sampled for such purposes. In cases of badly decomposed and/or skeletonized remains, analyses of collected carrion-feeding insects may provide the most accurate qualitative sources of toxicological information. Thus, this study pointed out that care must be taken in interpretations of blowflies' developmental patterns in cases where drugs or toxins may be involved in ases.

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