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THE USE OF LC-MS/MS TO DETECT NORDAZEPAM AND OXAZEPAM IN *CALLIPHORA VICINA* LARVAE

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OBJECTIVES

- *To develop a sensitive LC-MS/MS method for the detection and quantification of Nordazepam and Oxazepam in Calliphora vicina larvae (Diptera: Calliphoridae).*
- *To investigate the effect of Nordazepam and Oxazepam on the development of post-feeding larvae and pupae and hence, any possible implications for the determination of postmortem interval.*

INTRODUCTION

- In addition to their use in the estimation of postmortem interval, insects may serve as reliable alternate source for toxicological analyses in the absence of tissues and fluids normally taken for such purpose.
- To date, a variety of compounds have been measured in fly larvae and pupae using different analytical procedures i.e. (Radio-Immunoassay (RIA), Gas Chromatography (GC) and Thin-Layer Chromatography (TLC)). In these studies a minimum of 1g (approximately 20 larvae) was needed to detect the toxic compound.
- In this study we used LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) to detect the benzodiazepine Nordazepam and its metabolite Oxazepam, in single larvae of the *Calliphora vicina*. Benzodiazepines are prescribed for the symptomatic treatment of anxiety and sleep disorders. They are frequently encountered in postmortem blood analysis (suicide or accidental deaths).
- In addition, we compared the development of post-feeding larvae and pupae fed on different concentrations of Nordazepam.

EXPERIMENTAL CONDITIONS

Study design

Flies and larvae were from a stock colony of *Calliphora vicina* maintained in an environmental chamber at 18-24°C and 60-70 % humidity with cyclical artificial lighting simulating 16 h daylight and 8 h darkness.

Larvae were reared on artificial food (beef heart) spiked with a range of concentrations of Nordazepam (Table 1). Post-feeding larvae were harvested from day 4 till day 8. Thirty larvae were boiled and conserved (in a mixture of ethanol and acetic acid) prior to measurement of length. Another 30 were used for toxicological analysis. These were weighed and then killed, by freezing to -20°C. The larvae were stored at -20°C until analysis.

Sample	Target Concentration (µg/g)*
Control	0
Nor I (human therapeutic dose)	0.5
Nor II (human lethal dose)	1
Nor III (2x human lethal dose)	2

*Table 1: Target concentration of Nordazepam in larval food. *Concentrations expressed in µg/g food.*

Sample preparation

Individual larvae and pupae samples were prepared for LC-MS/MS as follows; the sample was transferred to a vial containing 500µL water and vortex-mixed thoroughly. One millilitre of acetonitrile (containing deuterated internal standards) was then added and the samples mixed for a further minute. The mixture was evaporated to ~100µL and then filtered. A 10µL aliquot was analysed using LC MS/MS.

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LC-MS/MS

LC Conditions

HPLC System: Waters Alliance 2690
Column: Zorbax SB–phenyl column (2.5x150mm, 5µm)
Mobile Phase: A=10:10:80 acetonitrile:methanol: 20mM ammonium acetate
B=95:5 acetonitrile: 20mM ammonium acetate

Time (min)	A(%)	B(%)	Curve Number
0	100	0	1
0.5	75	25	1
8	40	60	7 (concave)
11	40	60	6 (linear)
12	100	0	1
15	100	0	1

Flow Rate: 0.25mL/min
Injection Volume: 10µL

MS Conditions

Mass Spectrometer: Micromass Quattro *Ultima*™ triple quadrupole
Ionisation Mode: ES positive Ion
Capillary Voltage: 3kV

Table 2: MRM transitions and conditions for them LC-MS/MS analysis of Nordazepam and Oxazepam. Deuterated analogues were also included as internal standards.

RESULTS

All larvae, pupae and food spiked with Nordazepam were positive for the drug, whereas all control samples were negative. Figures 1 and 2 show the larvae Nordazepam and Oxazepam concentrations from days 4 - 8. Figure 3 shows the MRM chromatograms obtained following the LC-MS/MS analysis of a control larva and a nordazepam positive larva.

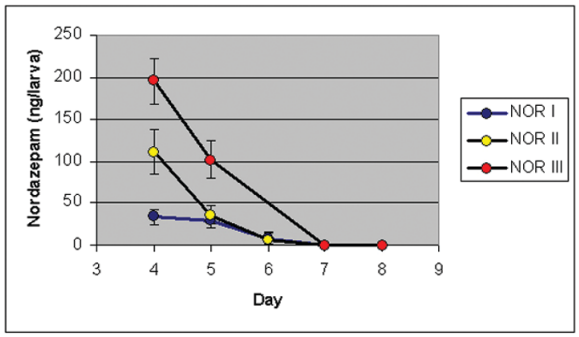

Figure 1: Concentration of Nordazepam in larva reared (for 4-8 days) on foodstuff spiked with Nordazepam. Mean concentrations are plotted (± 1SD).

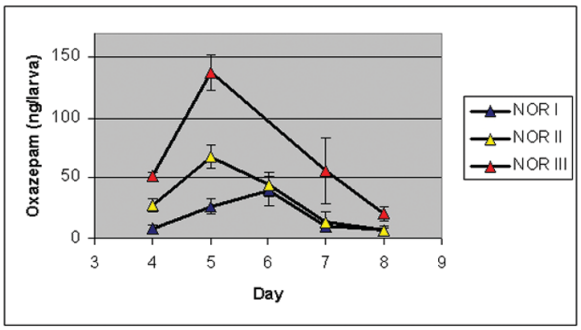

Figure 2: Concentration of Oxazepam in larva reared (for 4-8 days) on foodstuff spiked with Nordazepam. Mean concentrations are plotted (± 1SD).

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
Nordazepam	271	140	80	25
Nordazepam-d5	276	140	60	25
Oxazepam	287	241	60	26
Oxazepam-d5	292	246	80	26

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Peak concentrations of Nordazepam were measured on day 4 for NOR I, II and III, followed by a precipitous fall of larval Nordazepam concentrations. From day 7, Nordazepam was not detectable in a single larva.

Peak concentrations of Oxazepam were measured on day 5 for NOR II and III and at day 6 for NOR I. Low concentrations of Oxazepam were still measured at day 8. In this study, two patterns of development were observed; the post-feeding larvae fed on Control, NOR I and NOR III food regime developed at approximately the same rate and each demonstrated wandering-phase behaviour at day 6, pupation at day 8 and emerging of adult flies at day 18.

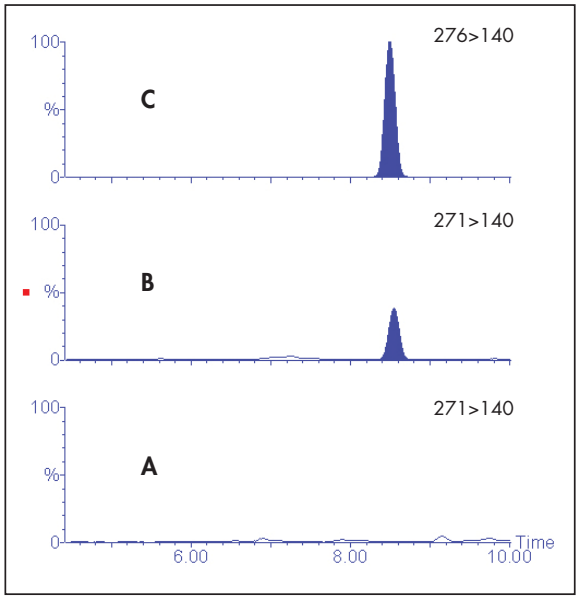

Figure 3: MRM chromatograms obtained with the analysis of larvae that were reared on artificial foodstuff spiked with Nordazepam at 0 and 1µg/g (A and B respectively). Figure C shows the MRM chromatogram for the internal standard i.e. Nordazepam d5.

In contrast, the development of larvae fed with the NOR II regime was 1 day later in all stages.

Post-feeding larval length is shown in Table 3; no significant differences were observed.

	Day 4	Day 5	Day 6	Day 7	Day 8
Control	16.7	17.8	15.2	15.8	15.9
NOR I	16.9	17.7	16.3	15.6	15.2
NOR II	17.2	17.3	16.7	15.5	15.8
NOR III	16.9	17.1	16.9	15.8	15.1

Table 3: Mean post-feeding larval length (mm)

	Day 4	Day 5	Day 6	Day 7	Day 8
Control	69.5	84.5	78.2	86.2	71.4
NOR I	73.4	89	78.5	87.5	80.5
NOR II	110.8	105.8	101.7	96	92.9
NOR III	82.5	83.5	83.5	84	83.1

Table 4: Mean post-feeding larval weight (mg)

Post-feeding larval weight is shown in Table 4: although no significant differences were seen in larvae reared on Control, NOR I and NOR III food regimes, the mean weight of larvae fed on NOR II was significantly higher. This observation was also confirmed in a second rearing experiment.

DISCUSSION AND CONCLUSIONS

We have developed a method that allows the detection of Nordazepam and its metabolite Oxazepam in single larvae. Larval drug concentrations showed a stepwise increase with increasing drug concentrations in the foodstuff. It was clear that Nordazepam was metabolized to Oxazepam, which was still detectable at day 8. Nordazepam was detectable until day 6. Control maggots were negative.

No differences were seen on the post-feeding larval length, but differences in post-feeding larval weight and development were seen in the NOR II larvae. The reason of this disturbance is not yet understood, but is presumably because larval physiology is disturbed to a greater extent by this drug level. This study indicates that an estimation of the postmortem interval based on the length of the

post-feeding larvae of *Calliphora vicina*, which have fed on tissues containing Nordazepam, will have no error. However an error, of up to 24 hours, can be made if the estimation is based on duration of larval and puparial stages.

FUTURE AIMS

- To valid this method for other Benzodiazepines
- To apply this method to other Diptera *(Calliphoridae, Muscidae, Sarcophagidae)*

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