

An LC/MS/MS Method for the Simultaneous Determination of Diazepam and its Metabolites in Fly Larvae Sampled From Diazepam Spiked Porcine Liver

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INTRODUCTION

Forensic entomology is a recognised method of estimating post-mortem interval, but relatively little research has examined the use of larvae in forensic toxicology. Forensic entomotoxicology includes the study of the effects of drugs and toxins on the development rate of carrion-feeding insects, and the use of these as alternative sample matrices in the absence of other samples. Analysis of living material, such as larvae, offers a number of technical advantages for drug detection over putrefied human remains. The extraction of drugs from larvae is the same as that from tissue, however, no emulsion is formed, whereas this is not always the case with human tissue. There is also less contamination observed from endogenous substances, which is particularly problematic with putrefied human remains. Larvae are usually present in abundance on decomposed bodies and sampling is often a relatively straight forward procedure.

To date, most forensic entomotoxicological studies have concentrated on opiates rather than commonly prescribed drugs, such as benzodiazepines. LC/MS/MS is becoming widely used in toxicology laboratories and is an established technique for screening and quantifying benzodiazepines. A rapid, sensitive and selective LC/MS/MS method for the identification of diazepam and its metabolites; desmethyl diazepam, oxazepam and temazepam in liver and fly larvae sampled from spiked porcine liver is described.

Diazepam, first approved for usage in the early 1960s, is one of the most frequently prescribed drugs of the benzodiazepine group. Its uses include; treatment of anxiety and anxiety related insomnia, muscle relaxant, anti-epileptic and pre-operative sedative. The frequency with which it is prescribed, coupled with the fact that regular, long-term use can lead to physical and psychological dependence, means that diazepam and its metabolites are regularly detected in post-mortem samples, and often in conjunction with illicit drugs. Diazepam has a high oral bioavailability, with peak blood levels occurring within 1 to 2 hours of administration. It is metabolised to its principal active metabolite, desmethyl diazepam, by N-demethylation. Diazepam and desmethyl diazepam undergo 3-hydroxylation to form temazepam and oxazepam, respectively, which are also active. Whilst desmethyl diazepam accumulates during chronic dosing, temazepam and oxazepam do not accumulate to any significant extent.

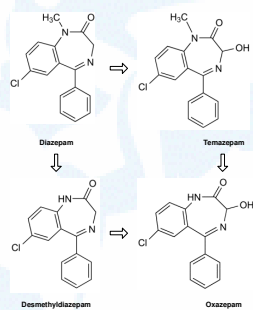


Figure 1. The Chemical Structures of Diazepam and its Metabolites.

EXPERIMENTAL

Calliphora vicina larvae were reared on minced porcine liver containing 0, 4, 9, 10 and 21mg/kg diazepam. The temperature was maintained at 20°C and fresh food stuff was introduced as required. Larvae (n=10) were harvested at various stages of development (days 4, 5, 6, 8, 10 and 12) and analysed for drug content by LC/MS/MS. The harvested larvae were initially transferred to a piece of damp cotton wool and further incubated for 24 hours after removal from the food source. This starvation period was to allow gut clearance. Drug levels detected are therefore representative of absorption into the larval tissues. The larvae were then sacrificed by immersion in boiling water and stored frozen at -20°C. Prior to analysis the larvae were washed with copious amounts of deionised water, to remove any remaining foodstuff and excretory waste, and then dried on filter paper. They were individually weighed, ground down with a pestle and mortar and diluted one part in ten with deionised water.

Materials

Individual drug standards of diazepam, desmethyl diazepam, temazepam and oxazepam were obtained from Sigma-Aldrich (Poole, Dorset, England). Diazepam-D5 (1mg/mL in methanol) was obtained from LGC Promochem (Middlesex, U.K.). HPLC grade acetonitrile and methyl-tert-butyl-ether (MTBE) were purchased from Rathburns Chemicals Limited (Walkerburn, Scotland). Ammonium formate and potassium phosphate was obtained from BDH (Poole, Dorset, England). Deionised water was prepared on site (ELGA Limited).

Extraction

The method involves liquid-liquid extraction of liver/larvae homogenate (100µL) into MTBE (1mL) at pH 7.0 (phosphate buffer, 250µL), after the addition of diazepam-D5 as the internal standard. The samples were mixed and then centrifuged. The upper organic phase was evaporated to dryness and reconstituted with 80% methanol (250µL). The extracts were then transferred to autosampler vials ready for analysis.

HPLC Conditions and MS Parameters

The HPLC equipment consisted of a Perkin Elmer PE200 series autosampler (injection volume, 25µL) and pump. A Sciex API2000 triple quadrupole mass spectrometer equipped with a turbo-ion spray interface maintained at 300°C was used for detection. An Alltech, Alltima C18 (150mm x 2.1mm, 5µm) column was maintained at 50°C in a Shimadzu CTO-10A column oven. The mobile phase, methanol-water (80:20, v/v), supplemented with ammonium formate to achieve a final concentration of 5mM, was pumped at 0.25mL/min. The method was run in positive ionisation mode and set to detect the precursor and product ions of diazepam (m/z : 284.9/154.0), desmethyl diazepam (m/z : 270.9/165.0) temazepam (m/z : 301.0/254.9) oxazepam (m/z : 286.9/241.1) and diazepam-D5 (m/z : 290.0/198.0). The total run time was 4.0 minutes.

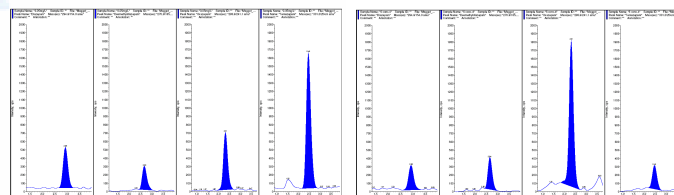


Figure 2. Chromatograms obtained for a 0.5mg/L Calibrator (left) and those for larvae harvested from the 21mg/kg containing liver at day 6 (right).

RESULTS AND DISCUSSION

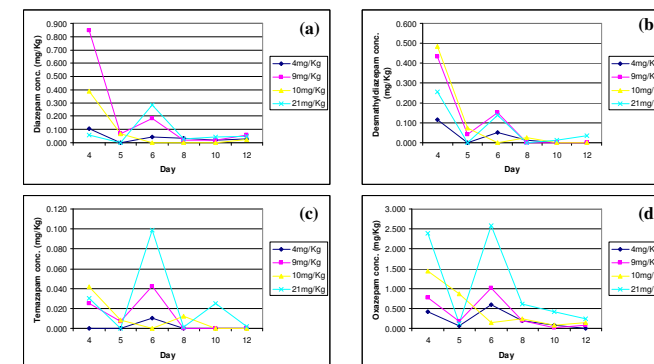


Figure 3. Comparisons between the measured diazepam (a), desmethyl diazepam (b), temazepam (c) and oxazepam (d) concentrations in larvae reared on 4, 9, 10 and 21mg/kg diazepam containing porcine liver over time. Each point represents the mean of 10 larval analyses.

Oxazepam is the most abundant metabolite detected at all time points and at all drug liver concentrations. With the exception of those larvae reared on liver containing 10mg/kg diazepam, a peak in drug and metabolite concentrations can be seen at day 6 and this correlated with a peak in larval weight and length.

CONCLUSION

We describe here a method for the simultaneous quantification of diazepam and its metabolites in larvae. The method comprises a simple extraction followed by LC/MS/MS analysis. The method was sufficiently sensitive to allow the detection of diazepam and its metabolites in a single larvae.

The concentration of their food source did not correlate with the concentration of diazepam or metabolites detected in individual larvae sampled at the designated time intervals. The lack of a simple relationship between larval and liver concentrations suggest that *C.vicina* larvae may not represent a quantitative measure of tissue concentrations. Larvae appear to metabolise and eliminate drugs with varying levels of efficiency and a decrease in concentration can be seen in non-feeding larvae preparing for pupariation. Diazepam did not significantly alter larval development or post-mortem interval when compared with those reared on blank liver.

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