An HPLC-MS-MS Method for the Simultaneous Determination of Diazepam and its Metabolites in Liver and in Fly Larvae Sampled from a Putrefied Cadaver

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BACKGROUND

The value of forensic entomological studies in the estimation of post-mortem interval is widely accepted. However, to date, the field of forensic entomotoxicology has remained largely under-investigated. Forensic entomotoxicology, the study of drugs and poisons in insects feeding on decomposing bodies, 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. It is possible that secondary bioaccumulation of drugs in larvae may also occur. Larvae are usually present in abundance on decomposed bodies and sampling is often a relatively straightforward procedure. A sensitive and selective HPLC-MS-MS method for the identification of diazepam and its metabolites in liver and in fly larvae sampled from a putrefied cadaver is described.

Diazepam, first approved for use 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, desmethyldiazepam, by N-demethylation. Diazepam and desmethyldiazepam undergo 3-hydroxylation to form temazepam and oxazepam, respectively, which are also active. Whilst desmethyldiazepam accumulates during chronic dosing, temazepam and oxazepam do not accumulate to any significant extent.

Figure 1. 10mg Generic Diazepam.

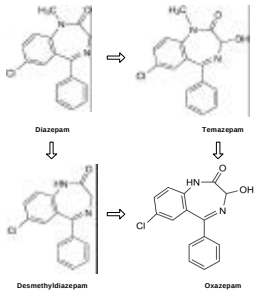

Figure 2. The Chemical structures of Diazepam and its Metabolites.

CASE HISTORY

On the 11th May 2004, police were called to the residence of the deceased by neighbours complaining of an unpleasant smell. Upon entry, the police found the putrefied cadaver of a 44 year old woman, covered with hundreds of larvae. The deceased had a history of i.v. drug abuse and was surrounded by a number of empty methadone bottles. The post-mortem examination was carried out on the 13th May 2004 and both liver and larvae samples were collected for toxicological analysis.

EXPERIMENTAL

Samples

Liver: Liver was sampled from the right lobe of the cadaver and homogenised (using an Ultra-Turrax homogeniser) with de-ionised water to produce a final dilution factor of 1 in 10.

Larvae: Larvae, identified as belonging to the Calliphora vicina fly and staged in the 3rd instar, were randomly collected from infested sites and were pooled. They were washed with copious amounts of de-ionised water to remove any exudation and transudation fluids and then dried on filter paper. The larvae were homogenised with de-ionised water to produce a final dilution factor of 1 in 4.

Figure 3. Blow Fly Larvae (Family Calliphoridae).

Materials

Individual drug standards of diazepam, desmethyldiazepam, temazepam, oxazepam and prazepam were obtained from Sigma-Aldrich (Poole, Dorset, England). HPLC grade acetonitrile and methyl-tert-butyl-ether (MTBE) were purchased from Rathburns Chemicals Limited (Walkerburn, Scotland). Potassium phosphate was obtained from BDH (Lutterworth, Leicestershire, England). De-ionised water was prepared on site (ELGA Limited).

Extraction

The method involved liquid-liquid extraction of 100µL liver/larvae homogenate into MTBE (1.5mL) at pH 7.0 (phosphate buffer, 250µL), after the addition of prazepam (0.1mg/L, 100µL) as the internal standard. The samples were mixed (approx. 5 minutes) and then centrifuged (3500rpm, 5 minutes). The upper organic phase was evaporated to dryness and reconstituted with 250µL of 80% methanol. 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, 10µL) and pump. A Sciex API2000 triple quadrupole mass spectrometer equipped with a turbo-ion spray interface maintained at 300ºC was used for detection. A Supelcosil LC-18-DB 15cm x 4.6cm, 5µm column was maintained at 50ºC in a Perkin Elmer PE200 peltier column oven. The mobile phase, methanol-water (85:15, v/v), supplemented with ammonium acetate to achieve a final concentration of 2mmol/L, was pumped at 1mL/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), desmethyldiazepam (m/z: 270.9/165.0) temazepam (m/z: 301.0/254.9) oxazepam (m/z: 286.9/241.1) and prazepam (m/z: 290.0/198.0). The total run time was 4.0 minutes.

RESULTS

The diazepam, desmethyldiazepam, temazepam and oxazepam concentrations observed were 0.16, 0.40, 0.04 , 0.12 and 3.10, 4.40, 0.50 and 0.27mg/Kg for the larvae and liver homogenates respectively. The liver concentrations are consistent with therapeutic use of diazepam.

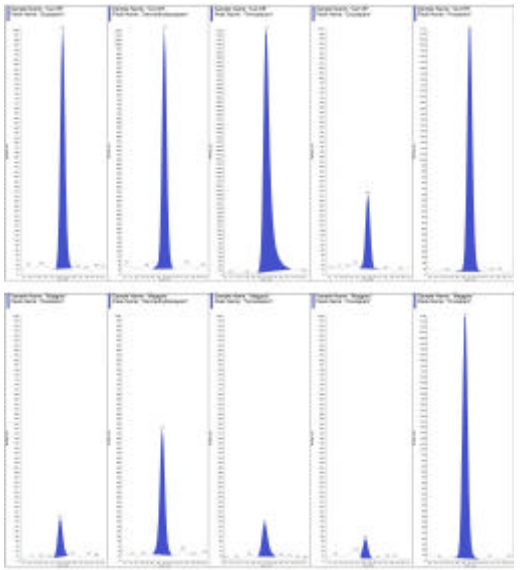

Figure 4. Chromatograms illustrating the results of initial benzodiazepine screening (0.1mg/L Cut off - Top) (Maggot Homogenate 1 in 4 dilution - Bottom).

CONCLUSION

The method described was found to offer both rapid and reliable analysis of benzodiazepines in fly larvae and post-mortem liver. It is unlikely that any robust quantitative relationship between larvae drug concentrations and those present in human tissues could be established. Larvae drug concentrations are likely to be affected by variables such as location of feeding, the drug or drugs involved, larvae developmental growth stage and a variety of other environmental factors. However, this study has demonstrated that in the absence of traditional toxicological specimens, such as blood and urine, larvae analysis may prove a useful qualitative alternative, particularly in cases where significant putrefaction has occurred. In addition as the presence of drugs may alter the rate of larvae development, entomotoxicology could provide an additional parameter for assessing the accuracy of post-mortem interval estimates.