The Determination of Chloralose Residues in Poisoned Birds of Prey By LCMSMS and LC-oaToFMS

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AIM:

- To develop a novel LC-MS method that facilitates the quantitative determination of chloralose residues present in animal tissues.
- To evaluate the utility of LC-oaToFMS for the on-line accurate mass measurement of pesticide residues

INTRODUCTION:

Chloralose (fig. 1) is a pesticide approved for use to control mice (indoors only) and, under special license, for the control of specific birds for public health reasons. Unfortunately, it has a more sinister use to illegally poison birds of prey and other animals. The determination of residues of a variety of pesticides including chloralose in suspected poisoning incidents is an operational requirement of the Wildlife Incident Investigation Scheme[1] (WIIS). The scheme was set up by UK government to investigate deaths of wildlife, beneficial insects, livestock and companion animals where pesticide poisoning might be involved.

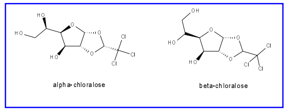

Figure 1. Chloralose [(R)-1,2-O-(2,2,2-trichloroethylidene)-α-D-glucofuranose]. $C_8H_{11}Cl_3O_6$ RMM = 308. Occurs as a mixture of two isomers α and β, ca. 85% and 15%, respectively.

Analytically supported diagnosis of poisoning is an important element of incident investigation, that can be allied with field information to attribute the cause of the incident to either approved use, mis-use or deliberate abuse of a pesticide. The determination of chloralose residues in extracts from animal tissues and/or digestive tract material has traditionally been based upon gas chromatographic procedures that involve extensive clean-up and derivatisation steps, which are arduous and time-consuming. Here we report upon the development, experimental considerations and benefits of LCMS based methodology for the quantitative analysis of chloralose in crude extracts (i.e. no sample clean up) from birds of prey (fig. 2).

Figure2. Common Buzzard (Buteo buteo), a frequent casualty of the illegal use of pesticides.

EXPERIMENTAL:

HPLC Method (Agilent 1100)

Column: Hypersil C18 BDS 3µm (100 x 4.6 mm I.D.)
Mobile Phase: Methanol / 10mM aqueous ammonium acetate pH 4.5 (55/45 v/v)
Flow-rate: 0.5 ml/min
Temperature: 35°C
Injection Volume: 20µl

Mass Spectrometry Method (Micromass Quattro Ultima)

Acquisition: Electrospray negative ionisation
Multiple reaction monitoring (MRM)
Collision gas: Argon
Data system: Mass Lynx 3.4

Analytical Procedure

Extraction: Liver tissue - Maceration in methanol
Digestive tract material - Tumbling in methanol
Clean-up: Not required, crude extract diluted with mobile phase and filtered
Detection: LCMSMS (m/z 307 + m/z 309 → m/z 161)
Confirmation: LCMSMS (m/z 307 + m/z 309 → m/z 189)

GC-based techniques (electron-capture detection) usually involved solid phase extraction clean-up steps, derivatisation to the trimethylsilyl adduct, and possibly off-line GCMS confirmation.

RESULTS & DISCUSSION:

Optimum MS parameters for chloralose were established experimentally following direct infusion of a solution in methanol into the mass spectrometer. The mass spectrum obtained using negative ion electrospray contained an intense $[M-H]^-$ molecular anion isotope group (fig. 3a). Two of these ions, at mass:charge (m/z) 307 and m/z 309, produced similar product-ion mass spectra when subjected to collision-induced dissociation using argon (figs. 3b and 3c). The m/z 307→ m/z 161 and m/z 307→ m/z 189 transitions correspond to the loss of $C_2H^{35}Cl_3O$ and $CH^{35}Cl_3$ neutral moieties, respectively. Whereas, m/z 309 → m/z 161 and m/z 309 → m/z 189 transitions correspond to the loss of $C_2H^{35}Cl_2{}^{37}ClO$ and $CH^{35}Cl_2{}^{37}Cl$, respectively.

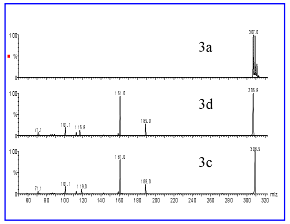

Figure3. Negative ion electrospray mass spectra:
Molecular anion isotope group(3a)
Product-io nm/z 307 (3b)
Product-ion m/z 309 (3c)

Chloralose was amenable to reversed-phase HPLC using simple binary mixtures of either acetonitrile or methanol with water, however the native chromophore offered extremely poor sensitivity for spectroscopic detection over the target concentration range i.e. 0.02 – 1.3mgml^{-1}. Optimised LCMS and LCMSMS methods were tested using solvent and matrix-matched standards and fortified matrix extracts. LCMSMS afforded superior sensitivity and selectivity and was adopted for subsequent experimental refinements i.e. m/z 307 + m/z 309 → m/z 161 summed transitions were used for screening purposes. Post-column flow splitting (ca. 15-20µl min^{-1} to the mass spectrometer) was also found to reduce ion-source maintenance requirements, inevitable when handling large numbers of 'dirty' samples, without compromising sensitivity.

A specific set of HPLC isocratic elution conditions capable of separating the two isomers was determined and the most suitable extraction solvent (methanol) was identified using LCMSMS detection. Figure 4 illustrates successful separation of the two isomers and how tissues extracted with methanol produced better defined peak shapes than those extracted with acetonitrile, although both solvents were able to satisfy extraction criteria defined by recovery requirements

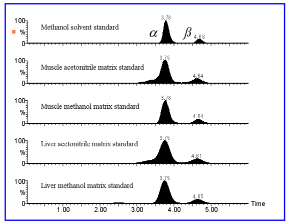

Figure 4. Reconstructed ion chromatograms illustrating effect of solvent (precursor ions m/z 307+ m/z 309 → product-ion m/z 161), for chloralose standards (1.5 ug/ml). Matrix concentrations equivalent to 0.025gml^{-1}.

The calibration obtained from standards prepared in pure solvent was found to be linear over the range 0.02 – 1.3µgml^{-1} and complementary experiments performed using standards prepared in a variety of matrix extract solutions indicated that calibration was virtually free from adverse matrix effects (fig. 5).

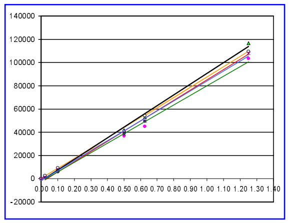

Figure 5. Calibration curves obtained for chloralose assay.
— Methanol solvent standard,
— Liver methanol matrix,
— Liver acetonitrile matrix,
— Muscle methanol matrix,
— Muscle acetonitrile matrix.
Other details as in figure 4.

Recovery experiments on extracts from tissues fortified with diferent amounts of chloralose were performed using calibration against standards prepared in unadulterated pseudo-matrix extract solution i.e. from chicken liver or muscle tissue. Crude extracts were simply diluted using mobile phase and filtered (0.45mm PTFE syringe filters) prior to analysis. The recoveries of chloralose from the fortified tissues satisfied internal QA requirements and were comparable with determinations carried out using gas chromatography with electron-capture detection (GC-ECD). Recoveries are shown in table 1.

Matrix (technique)	Fortification level mg/kg	Mean recovery (%)	Low (%)	High (%)	CV (%)	n
Chicken muscle (LCMSMS)	236.6	88.5	81	95	6.0	11
Chicken muscle (LCMSMS)	29.3	87.2	73	102	10.7	10
Chicken liver (LCMSMS)	29.3	81.3	72	93	7.4	15
Chicken liver (LCMSMS)	5.0	90.7	85	99	5.5	6
Chicken muscle (GC-ECD)	200	89.3	72	110	11.6	23
Chicken liver (GC-ECD)	25	80.2	73	91	6.4	16
Chicken liver (GC-ECD)	5	75.9	70	88	8.0	9

Table 1. Recovery of chloralose from fortified liver and muscle tissues using LCMSMS & GC-ECD.

The routine limit of detection was determined to be approximately 0.007mgmL^{-1} (signal:noise = 3:1), which is equivalent to 0.3mgkg^{-1} chloralose in liver tissue (fig. 6). This is well below the usual residue range of 5-130mgkg^{-1} in liver tissue associated with fatal poisoning.

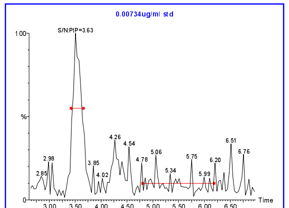

Figure 6. Determination of practical limit of detection for assay. Response from a chloralose standard at 0.00734 ug/ml prepared in muscle matrix extract (residue equivalent to 0.3 mg/kg in liver tissue).

Whenever a positive incident is indicated, confirmation of residues is a mandatory requirement. This was achieved by the simultaneous monitoring of the alternative structurally diagnostic transition i.e. m/z 307 + m/z 309 → m/z 189.

Examples from two separate incidents involving the suspected illegal poisoning of buzzards using this novel method are shown in figures 7 and 8, and demonstrate the success of this approach for the quantitative detection of chloralose residues extracted from buzzard gullet contents and buzzard liver. The turnaround time associated with GC-ECD analysis would have been ca 12 hrs, however this was reduced to ca 4hrs using LCMSMS, a saving of over 60%.

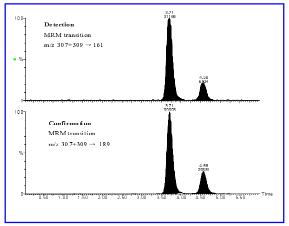

Figure 7. Detection and confirmation of a chloralose residue (1203 mg/kg) in a sample of material recovered from the gullet of a buzzard.

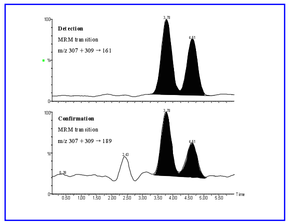

Figure 8. Detection and confirmation of a chloralose residue (5.2 mg/kg) in liver tissue from a buzzard.

ACCURATE MASS MEASUREMENT STUDIES:

The potential of Liquid Chromatography – orthogonal acceleration Time of Flight Mass Spectrometry (LC-oaToFMS) technique for:

1. the identification of unknown residue compounds
2. the screening of target pesticides
3. confirmation purposes

has been evaluated following the analysis of extracts of liver from a buzzard and a red kite.

Extracts taken from liver tissue that had been deliberately 'spiked' with chloralose (ca. 30mgkg^{-1}) were submitted as 'unknowns' and analysed by LC-oaToFMS (LCT Micromass UK Ltd). The instrument was operated at 5000 resolution (Full Width Half Maximum) in negative ion electrospray mode. The elevated resolution of oa-ToFMS allowed on-line accurate mass measurement of all anions associated with α and ß-chloralose chromatographic peaks using the full-scan function (i.e. mass spectral data was collected over a mass range of 50-500Da during data acquisition).

Figure 9 contains the background-corrected mass spectrum corresponding to the chromatographic region of interest and the elemental composition report (inset) relating to the buzzard extract. The report contains candidate empirical formulae and theoretical masses adjacent to the measured mass of the 'unknown' and within an error window set at 5.0mDa.

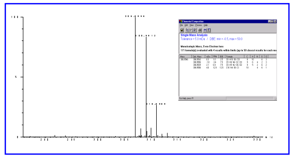

Figure 9. Background corected LC-oaToFMS mass spectrum of α Chloralose (Courtesy of Micromass UK Ltd.)

Table 2 displays a more comprehensive list of accurate mass measurements that incorporate the ^{37}Cl isotope and relate to the red kite liver extract. Careful consideration of mass spectral features in combination with accurate mass measurements meant that it was possible to identify the 'unknown' peak(s) as chloralose (theoretical mass = 306.9543Da):

Measured Mass	% Relative Abundance	Theoretical Mass	Error* (mDa)	Error (ppm)**	Formula
306.9555	100.0	306.9543	1.2	3.9	$C_8H_{10}{}^{35}Cl_3O_6$
		306.9535	2.0	6.6	$C_9H_{10}{}^{35}Cl_2{}^{37}ClO_5$
308.9530	95.6	308.9513	1.7	5.4	$C_8H_{10}{}^{35}Cl_2{}^{37}ClO_6$
		308.9505	2.5	8.0	$C_9H_{10}{}^{37}Cl_3O_5$
		308.9569	-3.9	-12.6	$C_{10}H_7{}^{35}Cl_2O_7$
310.9520	27.4	310.9539	-1.9	-6.2	$C_{10}H_7{}^{35}Cl{}^{37}ClO_7$
		310.9492	2.8	9.0	$C_7H_{10}{}^{35}Cl_3O_7$
		310.9484	3.6	11.6	$C_8H_{10}{}^{35}Cl{}^{37}Cl_2O_6$
		310.9565	-4.5	-14.4	$C_7H_9{}^{37}Cl_2O_9$

*Error window = 5.0mDa **Error window = 15.0ppm Correct assignment

Table 2. Comprehensive set of accurate mass measurements obtained following LC-oaToFMS analysis of a red kite liver extract.

Buzzard extract = 306.9546 (error = 0.3mDa, 1ppm)
Red kite extract = 306.9555 (error = 1.2mDa, 3.9ppm)

These errors satisfy American Chemical Society Guidelines which state that the difference between measured and theoretical masses should not exceed 5ppm for the identification of compounds with relative molecular mass <1000Da[2].

CONCLUSIONS:

- A robust LCMSMS method has been successfully developed and deployed to provide a specific screening and confirmation tool for the quantitative determination of chloralose in wildlife incident investigations
- Turnaround time is significantly reduced (ca. 60%) compared to GC based techniques used previously
- Simple dilutions of crude extracts can be analysed directly
- There are no significant matrix effects on detection or calibration
- The limit of detection available exceeds the target specification
- On-line accurate mass measurement of ions was demonstrated using oa-ToF instrumentation and the technique offers a powerful complementary tool for the identification of unknown compounds and for the screening or confirmation of target pesticides

REFERENCES:

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2. JAMCS, 1999. Notice to authors of papers. *Journal of the American Chemical Society* 121: 7A-12A.