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Determination of the Phenoxyacid Herbicides MCPA, Mecoprop and 2,4-D in Kidney Tissue Using Liquid Chromatography with Electrospray Tandem Mass Spectrometry

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Abstract An analytical method was developed to determine the phenoxyacid herbicides 2,4-D, MCPA and mecoprop in kidney tissue from animals where poisoning is suspected. Samples were Soxhlet extracted using diethyl ether and the extracts cleaned-up using anion exchange solid phase extraction cartridges. Analysis was performed using liquid chromatography with negative-ion electrospray tandem mass spectrometry (LC-MS/MS). The method was evaluated by analysing control kidney samples fortified at 1 and 5 mg/kg. Mean recoveries ranged from 82 to 93% with relative standard deviations from 3.2 to 19%. The limit of detection was estimated to be 0.02 mg/kg.

Keywords Acid herbicides · Pesticide poisoning · Liquid chromatography · Mass spectrometry

For more than 25 years in England and Wales, suspected cases of deliberate or accidental pesticide poisoning of vertebrate wildlife, companion animals and honeybees have been investigated by the Wildlife Incident Investigation Scheme (WIIS) (Fletcher and Grave 1992). The Wildlife Incident Unit at the Central Science Laboratory performs the pesticide residue analysis of submitted samples, prioritising analyses with consideration to *post-mortem* findings and circumstantial evidence such as local pesticide use or history of abuse. Traditionally the focus has been on pesticides with high toxicity to vertebrates and honeybees – mostly anticholinesterase insecticides and anticoagulant rodenticides, although lower toxicity compounds such as alphachloralose and metaldehyde have also regularly been identified in poisoning incidents (Brown et al. 2005). Lately, however, interest in fungicides and herbicides has increased, partly as a result of rising concern over 'cocktail' effects and/or synergism (Thompson 1996), but also because occasionally fungicide and herbicide residues are found in WIIS samples, although the significance of such residues is often unclear.

In UK agriculture, total herbicide and fungicide use far exceeds the use of all other pesticides, including those most often reported as residues by the WIIS (Garthwaite et al. 2005, 2006). Although these classes of compounds are generally of very low toxicity to birds and animals, with rat LD_{50} values of several thousand mg/kg bodyweight, there are some notable exceptions where toxicity to rats is almost ten-fold higher. The phenoxyacid herbicides 2,4-D, MCPA and mecoprop-p (the R isomer of mecoprop) stand out as both heavily used and relatively toxic. Toxicity seems quite variable according to species. For example, rat LD_{50} values for 2,4-D are 639–764 mg/kg, whereas the LD_{50} for mice is 138 mg/kg (Tomlin 2006). Dogs seem particularly sensitive to poisoning by 2,4-D (Van Ravenzwaay et al. 2003; Timchalk 2004) with lowest reported LD_{50} data of around 100 mg/kg (Drill and Hiratzka 1953). Residue and toxicity data for these compounds in wild animals are scarce or non-existent. The WIIS provides a means by which possible lethal or sub-lethal effects can be monitored in 'real world' situations. In order to do this, a multiresidue analytical method was developed for 2,4-D, MCPA and mecoprop in kidney. These highly water-soluble herbicides have been shown to be almost exclusively excreted via the renal route, with highest tissue residues usually occurring in the kidney (Van Ravenzwaay et al. 2004; Aydin et al. 2005; Barnekow et al. 2001). Multiresidue methods for the

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determination of phenoxyacid herbicides have been described in a variety of sample types such as vegetation and plant products (Rimmer et al. 1996; Kuang et al. 2006), water (Wu and Lee 2006; Nilsson et al. 1998; Henriksen et al. 2001; Thorstensen et al. 2000), soil (Moret et al. 2006), plasma and urine (Dickow et al. 2001) and frog and fish tissues (Shin 2006). 2,4-D has also been detected in kidney following extraction by homogenisation, liquid/liquid partition, solid phase extraction (SPE) and LC analysis with UV detection (Aydin et al. 2005). Solvent intensive liquid/liquid partitions and/or derivatisation with hazardous reagents for gas chromatographic determination are often involved in published methods. The method reported in this paper dispenses with these steps, using a convenient SPE clean up and LC with mass spectrometric detection to provide a highly selective, yet simple, multiresidue method with good recoveries.

Materials and Methods

All reference substances were 96% purity or better. 2,4-D was obtained from QMx Laboratories (Great Halstead, UK). MCPA and mecoprop were obtained from Riedel-de-Haen (Sigma–Aldrich, UK). Methanol, ethanol and water were HPLC grade (Fisher, Loughborough, UK). Formic acid, ammonia solution (35%), ammonium acetate, acid-washed sand, sodium acetate and granular, anhydrous sodium sulphate were reagent grade (Fisher, Loughborough, UK). Diethyl ether was glass distilled grade (Rathburn Chemicals Ltd, Walkerburn, UK). Oasis MAX SPE cartridges (150 mg, 6 mL) were from Waters (Elstree, UK). Whatman cellulose Soxhlet thimbles (28 mm × 80 mm) were obtained from Fisher (Loughborough, UK).

Chromatography was performed using an Agilent 1100 Series binary HPLC pump, autosampler, and degasser (Agilent Technologies, Wokingham, UK) with an Atlantis dC_{18}, 50 mm × 2.1 mm i.d., 3 µm particle size analytical column (Waters Ltd, Elstree, Herts, UK) and a C_{18} Security Guard guard column cartridge, 4 mm × 2 mm (Phenomenex). Mobile phase solvent A was 1 mM ammonium acetate in water, and mobile phase solvent B was methanol. The mobile phase composition was initially 85% A and 15% B with a linear gradient to 10% A and 90% B over 4.5 min, remaining isocratic for a further 7.7 min before re-equilibration at the initial composition. Flow rate was 150 µl/min for the first 13 min, and then was increased to 250 µl/min for 7 min during column re-equilibration at the initial mobile phase composition, returning to 150 µl/min by the end of the 21 min run time. Injection volume was 7 µl and a switching valve was used to divert to waste the first 4.5 min of column eluent, to reduce contamination of the source by any unretained sample coextratives.

Tandem mass spectrometry was performed using an Applied Biosystems Sciex API 2000 triple quadrupole mass spectrometer (Toronto, Canada). All analyses were carried out using the electrospray source (TurboIonSpray™) at 300°C in negative-ion mode with capillary voltage set to −4,500 V and multiple reaction monitoring (MRM) acquisition. The ion transitions that were monitored are detailed below (Table 1) along with declustering potential (DP) and collision energy (CE) used. Dwell time was 100 ms per channel with a 5 ms interchannel delay. Nitrogen gas was used for collisionally activated dissociation (CAD) at a flow rate of two 'arbitrary units' (Sciex, au). Nitrogen was also used for curtain gas, 'Gas 1' and 'Gas 2' (Sciex) at flow rates of 40, 30 and 70 au.

Sub-samples of pig kidney (~5 g), known to be free from phenoxyacid herbicide residues, were weighed in mortars. The tissue was fortified with a mixture of the analytes in methanol at rates of 1 and 5 mg/kg (100 µl of a 50 or 250 µg/mL solution of the acids in methanol, respectively). Five replicates at each level were prepared. After fortifying, the samples were left for an hour at room temperature before extraction. Control samples were prepared in the same way as the fortified samples but without addition of the analytes.

Sand (5 g) and sodium sulphate (25 g) were added to the kidney tissue in the mortar and were ground with the pestle to obtain a free-flowing powder. The dry mixture was transferred to a pre-washed (by Soxhlet extracting for 3 h with diethyl ether) cellulose extraction thimble, which was Soxhlet extracted for 12–16 h with diethyl ether. The extract was then transferred to a 100 mL volumetric flask and made up to volume.

An aliquot of extract equivalent to 0.5 g of kidney (10 mL) was evaporated to dryness under nitrogen. The residue was redissolved in methanol with 2% ammonia (v/v) (1 mL). A Waters Oasis MAX cartridge was conditioned with methanol (5 mL) followed by water (5 mL). The sample solution was loaded onto the cartridge, followed by two sample tube rinses using methanol with 2% ammonia (v/v) (1 mL), and the eluates discarded. The cartridge was then washed with sodium acetate solution

Table 1 MS transitions monitored

Compound	Q1 ion (m/z)	Q3 ion (m/z)	DP (V)	CE (V)
2,4-D	219	161[a]	−18	−25
	221	163	−18	−25
MCPA	199	141[a]	−8	−20
	201	143	−8	−20
Mecoprop	213	141[a]	−20	−25
	215	143	−20	−25

[a] Transitions used for quantification

(5 mL, pH 7, 50 mM) and then ethanol (5 mL) and both eluates were also discarded. Finally the analytes were eluted from the cartridge with ethanol containing 2% formic acid (v/v) (10 mL). This solution was evaporated until just dry and redissolved in aqueous methanol (methanol:water, 50:50 (v/v), 5.0 mL for the 1 mg/kg level and 10 mL for the 5 mg/kg level). This solution was analysed directly by LC-MS/MS with matrix-matched calibration solutions.

Stock calibration solutions of the analytes were prepared from reference materials at a concentration of 1,000 μg/mL in acetone. These were then used to prepare solutions of the three phenoxyacid herbicides in methanol of 250 and 50 μg/mL. A range of calibration solutions from 0.05 to 0.75 μg/mL were prepared from dilutions in methanol. For LC-MS/MS analysis, matrix-matched calibration solutions in the same range were obtained by preparing extracts from control kidney tissue, as described above, except that the evaporated residue was dissolved in 5.0 or 10 mL of calibration solution rather than in pure solvent.

Results and Discussion

Average recoveries were good, in the range of 82–93% (Table 2), with acceptable variability for the intended application of the method. Chromatograms were free from interfering peaks (Fig. 1). Peak shape was good with little tailing. For simplicity, and compatibility with other analyses run on the same system, a standard, aqueous ammonium acetate/methanol gradient was used. Retention time drift was acceptable for ionisable analytes on a reverse-phase column in unbuffered mobile phase. The limit of detection (LOD), defined as a signal to baseline noise ratio of 3:1, was estimated by extrapolation to be about 0.02 mg/kg. MS response was linear over the range of calibrants tested (0.05–0.75 μg/mL). Average MS response for analytes in matrix-matched solutions was very similar to those in pure solvent when the final volume of analysed solution was 10 or 5.0 mL (equivalent to 0.05 g sample/mL and 0.1 g sample/mL, respectively), suggesting negligible effect of post-clean up sample components on ionisation.

Table 2 Mean recoveries of phenoxyacid herbicides from fortified kidney

Analyte	1 mg/kg (n = 5)		5 mg/kg (n = 5)	
	Average recovery (%)	RSD (%)	Average recovery (%)	RSD (%)
2,4-D	85	19	82	16
MCPA	89	12	88	7.9
Mecoprop	93	8.5	87	3.2

Phenoxyacid herbicides are formulated as various esters or salts. The presence of esters as residues as well as free acids would require a more complicated analytical method to either convert the esters to acids or to measure each ester and free acid separately. However, when 2,4-D 2-ethylhexyl ester was orally administered to rats, none of the ester was detected in the urine or blood. 2,4-D free acid was detected in both indicating that the ester is rapidly converted to the free acid (Frantz and Kropscott 1993). Similarly, MCPA ethylhexyl ester is rapidly excreted in the urine as the free acid following rapid hydrolysis in vivo (Van Ravenzwaay et al. 2004). Rats administered 2,4-D excreted 95% of the dose as free acid within 48 h (Schulze et al. 1985). Physico-chemical similarities between the three phenoxyacid herbicides make it reasonable to assume that mecoprop esters will have similar pharmacology to 2,4-D and MCPA, and that a method to detect just the free acid form of the analyte would be sufficient.

Due to the inability to distinguish between the two optical isomers of mecoprop without using an expensive chiral LC column, racemic mecoprop was used for method development instead of mecoprop-p, the isomer in most current formulations in the UK. It was assumed that a method that worked for the racemate would work for both individual isomers, too.

Lack of reported residues from fatally poisoned animals made informed selection of appropriate validation levels difficult. One published report was found where a dog, euthanased 25 h after a dose of 175 mg/kg 2,4-D, had a kidney residue of 271 mg/kg (Arnold et al. 1991). It was decided to validate at levels that were considerably lower than this (1 and 5 mg/kg) to allow some margin for detecting residues in species more sensitive to phenoxyacid herbicides than dogs, or for situations where more metabolism or excretion occurs before death, or when decomposition of residues occurs before a dead animal is found and *post-mortemed*.

Soxhlet extraction provides an exhaustive extraction procedure but is time-consuming, requiring several hours of refluxing. An alternative procedure involving vigorous agitation of the sample in either dichloromethane (DCM) or DCM and acetone (70:30 v/v) on a flat-bed shaker followed by filtration was tried, but gave low recoveries. Shaking with DCM only, gave recoveries between 17 and 31%. Adding acetone improved the recoveries to between 35 and 68%. In all cases, Soxhlet extraction gave highest recoveries.

In order to reduce solvent consumption and sample preparation time, SPE was investigated in preference to widely used liquid/liquid partition clean up methods. Polymeric anion exchange SPE cartridges were found advantageous in previous, unreported work with acidic analytes in animal tissue extracts. They have the

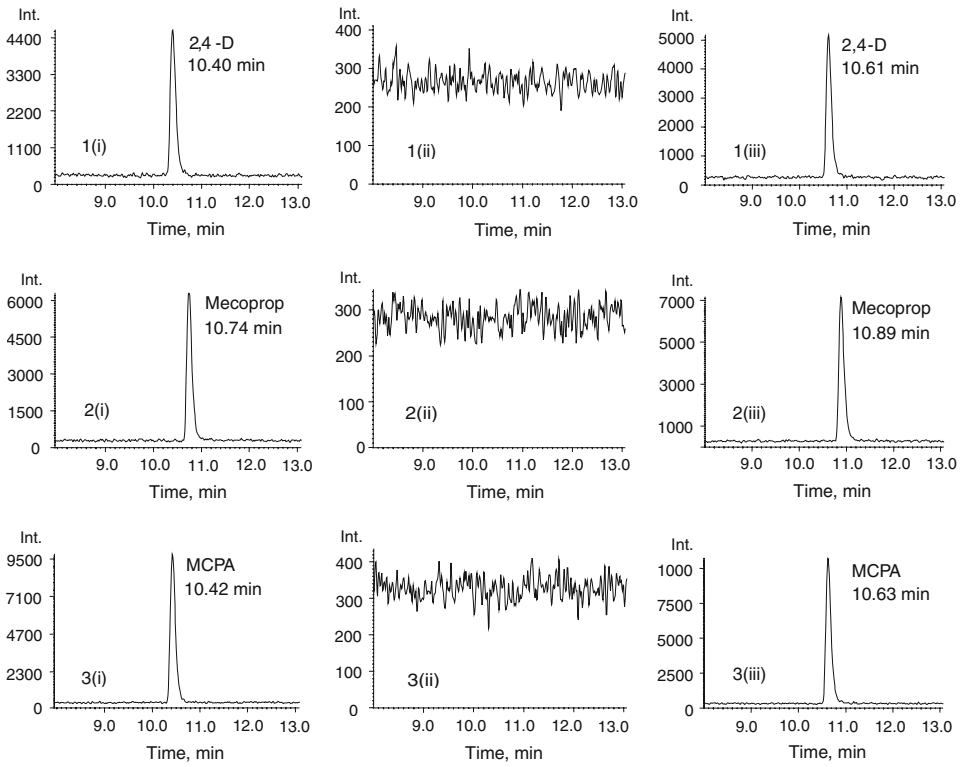

Fig. 1 Chromatograms of phenoxyacid herbicides. *1(i)* 0.1 μg/mL 2,4-D matrix-matched calibration solution, *1(ii)* blank kidney sample, *1(iii)* 1 mg/kg 2,4-D fortified kidney sample, *2(i)* 0.1 μg/mL mecoprop matrix-matched calibration solution, *2(ii)* blank kidney sample, *2(iii)* 1 mg/kg mecoprop fortified kidney sample; *3(i)* 0.1 μg/mL MCPA matrix-matched calibration solution, *3(ii)* blank kidney sample, *3(iii)* 1 mg/kg MCPA fortified kidney sample

advantages, when compared to traditional surface-coated silica adsorbents, of wider pH tolerance and higher capacity per weight of adsorbent, and they are less affected by running dry during use. These factors add robustness to the SPE procedure. The polymeric "back bone" supporting the cationic, quaternary amine groups gives the cartridges some reverse-phase functionality. The analytes were most reliably retained on the cartridge when loaded in basic pH conditions, then were eluted in acidic pH conditions. Phenoxyacid herbicide recovery was maximised by optimising pH and ionic strength of eluent. Between retention and elution of the analytes, the cartridge can be washed with neutral buffer solution then miscible organic solvents, which elutes neutral and basic coextractives retained by a reverse-phase mechanism. Ethanol was found to be better than methanol for this purpose, probably because it is slightly less polar than methanol. Comparison of evaporated sample solution residue weights before and after clean up showed that 86% of extracted material had been removed, which reduces contamination of LC column and MS source.

Gas chromatography with mass spectrometry (GC-MS) was considered initially but was abandoned in favour of LC-MS/MS. The analytes are too polar for direct GC analysis on typical stationary phases and so were converted to their methyl esters. This extra step in the procedure added complexity. Also, on both (5%-phenyl)-methylpolysiloxane and (50%-phenyl)-methylpolysiloxane stationary phases, peak tailing quickly became a problem and necessitated frequent column maintenance.

An LC gradient was devised with a higher mobile phase flow rate after elution of the analytes. This enabled complete column re-equilibration with good peak shape and retention of these acidic compounds within an overall analysis runtime of 21 min. The LC-MS/MS ion transitions and their optimum declustering potential (DP) and collision energy (CE) were established by infusion of calibration solutions. For all three compounds, there was only one suitable fragment ion. Although it is generally considered desirable to use a different fragment ion for confirmation, in this case the same fragment with a different chlorine isotope was used for the second transition (Cl-37 rather than Cl-35). The absence of any chromatographic interference from sample coextractives at these mass transitions makes isotope-based confirmation acceptable. MCPA and mecoprop also share Q3 fragment ions (Table 1), which is not unexpected given their structural similarity. However, it is evident from the relative retention times of these two compounds that there is no sign of cross-talk between channels at all, even with only a 5 ms inter-channel delay. Although not analysed as part of this study, the chromatography and mass spectrometry conditions used are likely to be suitable for other, similar acidic herbicides.

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