Food and Chemical Toxicology 59 (2013) 438–445

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Comparisons of metabolic and physiological changes in rats following short term oral dosing with pesticides commonly found in food

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ARTICLE INFO

Article history:
Received 12 October 2012
Accepted 21 June 2013
Available online 30 June 2013

Keywords:
Food safety
Metabolomics
Pesticide
Toxicology
Time course

ABSTRACT

^{1}H Nuclear Magnetic Resonance spectroscopy has been used to profile urinary metabolites in male Fischer F344 rats in order to assess the metabolic changes induced by oral exposure to two benzimidazole fungicides (carbendazim and thiabendazole) and two bipyridyllium herbicides (chlormequat and mepiquat). Exposure levels were selected to be lower than those expected to cause overt signs of toxicity. We then compared the sensitivity of the metabolomics approach to more traditional methods of toxicity assessment such as the measurement of growth and organ weights. Separate, acute exposure experiments were conducted for each pesticide to identify potential metabolic markers of exposure across four doses (and a control). Growth, organ weights and feeding/drinking rates were not significantly affected by any compounds at any dose levels tested. In contrast, metabolic responses were detected within 8 and 24 h for chlormequat and mepiquat, and after 24 h for carbendazim and thiabendazole. These results demonstrate the potential for the use of metabolomics in food toxicity testing.

1. Introduction

The majority of crops grown worldwide are routinely treated with a variety of pesticides throughout the growing season. The extent of pesticide usage varies with each crop and between countries but many compound classes are applied throughout the year. For example, herbicides are applied on arable crops in the UK with an average of three applications of four products and five active substances per year (Garthwaite et al., 1999). This leads to pesticide residues occurring in food products, albeit at low concentrations of each individual compound (Hernández-Borges et al., 2009). In recent years the increasing public concern over this issue has led to the need for tools to aid in the hazard identification and risk assessment of these chemicals to be clearly recognized (Miles and Frewer, 2001; Royal Commission on Environmental Pollution, 2003).

The risk posed to human health from pesticide residues in foodstuffs is currently assessed on a compound by compound basis using residue data from environmental monitoring in conjunction with toxicological information obtained from laboratory studies on model species (Bondy et al., 2004). However, these assessments tend to be aimed at finding the level that causes gross histopathological changes, such as organ damage, behavioural problems (such as the blinking response) or a reduction in feeding/drinking rates. These studies are clearly important but by focussing on overt toxicity they may miss subtle, low level effects on metabolism. Such metabolic changes may not directly reflect those seen at high levels of exposure (food toxicity) of individual pesticides since low levels of toxicant exposure may induce different changes than those induced by higher exposures but they have could be used to provide early warnings of possible toxicity prior to irreversible damage occurring; this would have great potential use in food safety assessment and regulation (Favé et al., 2009).

Metabolomics provides a quick and convenient technique to investigate potential toxicity in biofluids from any species (Griffin and Shore, 2007). The approach is particularly useful at confirming organ specific toxicity following the identification of metabolic markers in biofluids (Beckwith-Hall et al., 1998; Garrod et al., 2005). This method also has a large practical advantage over other omic/systems biology based technologies such as transcriptomics and proteomics in that metabolites are similar in the majority of species; thus, a fully annotated genome is not required for analysis and analytical methods are transferable between species

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http://dx.doi.org/10.1016/j.fct.2013.06.041

(Jones et al., 2008). Here, we have used ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy to analyse metabolic profiles from urine collected from rats (*Rattus norvegicus* – inbred Fischer F344 strain) exposed to single doses of one of four different, yet commonly used, pesticides which have been shown to occur in UK food products. The aim of this study was not to prove effects at real everyday food concentrations. The principle hypothesis under test was that it would be possible to develop metabolic markers of exposure and effect (relevant for assessment in higher mammals) that may, in future, enable early and reliable detection of systemic responses and health effects arising from such exposures.

2. Material and methods

2.1. Pesticide selection

Compounds were selected for this study on the basis of having; (a) been shown to occur in the UK food supply, (b) specific, but differing modes of action and (c) some evidence of exceedance of the Acceptable Daily Intake (see Section 2.2) in foodstuffs. The pesticides chosen were 2 benzimidazole fungicides; specifically carbendazim (CBZ) and thiabendazole (TBZ), and 2 bipyridyllium herbicides; specifically chlormequat (CMQ) and mepiquat (MPQ). These compounds are some of the most commonly found compounds in fruit, vegetables and cereals in national and co-ordinated monitoring programmes within the UK and Europe where doses above the ADI are sometimes reported (European Union, 2006).

2.2. Dose selection

Since the aim of the study was to detect metabolic responses before permanent physiological changes manifested the pesticide exposure levels were chosen to be high enough to cause a metabolic response but not to result in overt clinical signs of toxicity. The exception to this is the lowest dose of each compound where it was decided to use a dose that was relevant to the human exposure level, assessed via the human Acceptable Daily Intake (ADI) as used by the UK Food Standards Agency (FSA). The ADI is a measure of the quantity of a particular chemical in food that can be consumed on a daily basis over a lifetime without harm. Theoretically this level of exposure should have no effect. All ADIs were taken from the latest edition of the pesticide manual (Tomlin, 2009) and the final dose ranges used in this study are outlined in Table 1 and were approved by the FSA. The aim of the experiment was not to study the toxicological effects of these pesticides, which are already well studied (World Health Organization, 2005) but to investigate if metabolomics could be used give an early warning of effect at lower exposure levels than organ weight and feeding/drinking rates. Since the doses were not intended to relate directly to average food concentrations (but rather to determine if metabolomics could be used as early and sensitive detection of intakes exceeding ADI) they were selected between the ADI and levels causing any overt effects.

2.3. Animal handling and dosing

All experiments were approved by CEH ethics committee and conducted in accordance with the Home Office Guidelines for the Care and Use of Laboratory Animals (UK), which complies with the code of ethics of the world medical association (Declaration of Helsinki) for experiments involving animals.

Male Fischer F344 rats (8 weeks old) were obtained from Harlan (Bicester, UK) and maintained in constant conditions (21 °C, 12 h light/12 h dark) for 5 days prior to use in the study. During this period they were housed in groups in plastic cages with wood shavings as bedding material and had access to food (standard rat maintenance diet – RM1) in pellet form (Special Dietary Services; Witham, UK) and tap water *ad libitum*.

At the start of each exposure rats were randomly selected and weighed. The mean start weights were 200 g ±1.45 SD (194–206 g). They were then placed individually in metabolism cages where they again had free access to food (ground form of RM1) and water at all times. After 24 h they were given a single dose, via oral gavage, of one of four doses of pesticide, or sham dosed as controls. The dosing vehicle was corn oil for the benzimidazole fungicides and distilled water for the bipyridyllium herbicides. Sham dosed animals were given just water or corn oil. The volume of each dosing vehicle was 0.5–1 ml in all cases. Over the next 5 days urine samples were collected using metabolism cages. Thus, for each individual rat, urine was collected at a total of four time intervals, namely; the 24 h before dosing (pre-dose) as well as 0–8, 8–24 and 96–120 h post dosing. It is possible that any biomarkers observed using this method could be related to the actual consumption of the tested compounds and not specific with toxicity. However, this is unlikely since the controls (dosed with water/corn oil) did not respond in the same away as the animals dosed with pesticides.

The rats were weighed for a second time at the end of each experiment to determine their weight gain over the 5-day testing period. Food and water consumption were also measured for each period the rats were in the metabolism cages. At the end of the experiment the rats were sacrificed using cervical dislocation and the liver, kidneys, brain and testes were removed and weighed (see Figs. 1–3).

2.4. Experimental design

Due to availability of metabolism cages only 15 rats could be monitored individually at any one time. Rats were therefore split in batches and exposures staggered over 8 weeks, with both compounds from each pesticide class run at the same time (week 1, 2, 5 and 6 for CBZ/TBZ and week 3, 4, 7 and 8 for CMQ/MPQ) with common controls used for each pesticide class. Sodium azide was added to the urine collection pots to minimise bacterial degradation and all samples were stored at −80 °C immediately after collection.

2.5. ^{1}H-NMR analysis

All urine samples were analysed using ^{1}H NMR spectroscopy. Prior to NMR analysis, samples were thawed at 4 °C, spun at 447 g for 5 min and the supernatant collected. A 200 μl sub-sample of urine was then added to 400 μl of phosphate buffered deuterium oxide. The buffer consisted of 200 mM sodium phosphate, pH 7.4, containing 0.1% sodium azide (to minimise bacterial degradation) and 1.2 mM sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) (Cambridge Isotope Laboratories Inc., Hook, UK) to provide a chemical shift reference ($\delta = 0.0$) for the resulting spectra. This mixture was then centrifuged for 10 min at 704 g and all samples were then transferred to individual 5 mm NMR tubes (Sigma–Aldrich Gillingham, UK) prior to analysis (Griffin et al., 2007).

Samples were analysed using a 500 MHz AVANCE II + spectrometer (Bruker, Coventry, UK) operating at 500.13 MHz for the ^{1}H frequency using a 5 mm Broadband TXI Inverse ATMA probe. Spectra were collected using a solvent suppression pulse sequence based on a 1 dimensional NOESY pulse sequence to saturate the residual ^{1}H water signal (relaxation delay = 2 s, $t_1 = 3$ μs, mixing time = 150 ms,

Table 1
Dose range (mg/kg body weight) used for single dose acute toxicity testing.

Pesticide	Low dose*	Intermediate dose 1	Intermediate dose 2	High dose
Carbendazim (CBZ)	0.5 (*200)	100 (25 mg/kg(bw)/day)	375	750
Thiabendazole (TBZ)	0.1 (*400)	40 (9 mg/kg(bw)/day)	120	300
Chlormequat (CMQ)	0.05 (*1000)	50 (23 mg/kg(bw)/day)	75	100
Mepiquat (MPQ)	0.6 (*83)	50 (3000 ppm in diet ~ 240 mg/kg(bw)/day)	75	150

*Figures in parentheses indicate ratio of Intermediate dose 1/low dose.
Figures in italics represent the no observed effect levels (NOEL) for each pesticide.
Low dose where it was decided to use a dose that was relevant to the human exposure level, assessed via the human (UK) Acceptable Daily Intake (ADI) as used by the Food Standards Agency (FSA) in the UK (see Section 2.2).
Intermediate dose 1 – was aimed at being between 2 and 4 times higher than the estimated NOEL (given in italics in Table 1), which was achievable for CBZ, CMQ and TBZ. The only exception to this approach was for mepiquat as the suggested NOEL of 3000 ppm seemed unreasonably high, and the low dose was that previously tested in a pilot study.
Intermediate dose 2 – was selected as a dose in between the high and intermediate dose 1 from which it was thought that changes in metabolism would result.
High dose – These doses set using pilot studies to be as high as reasonably possible without causing unacceptable signs of toxicity (e.g. a blinking response).