Hair analysis used to assess chronic exposure to the organophosphate diazinon: a model study with rabbits

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The main purpose of the present study was to determine whether hair analysis would be a suitable method to assess chronic exposure of rabbits to the pesticide diazinon. A controlled study was designed, in which white rabbits of the New Zealand variety were systemically exposed to two dosage levels (15 mg/kg per day and 8 mg/kg per day) of the pesticide, through their drinking water, for a period of 4 months. Hair samples from the back of the rabbits were removed before commencing the experiment and at the end of the dosing period. Parallel experiments with spiked hair were carried out in order to design a simple and efficient method of extraction of diazinon from hair. The hair was pulverized in a ball mill homogenizer, incubated in methanol at 37 °C overnight, liquid–liquid extracted with ethyl acetate and measured by chromatography techniques (GC-NPD and GC-MS) for confirmation. The concentration of the diazinon in the hair of the exposed animals ranged from 0.11 to 0.26 ng/mg hair. It was concluded that there is a relationship between the administered dose and the detected pesticide concentration in hair. Finally, it seems that hair analysis may be used to investigate chronic exposure to the pesticide. Human & Experimental Toxicology (2003) 00, 1–6

Key words: chronic exposure; diazinon; GC-NPD; GC-MS; hair

Introduction

Diazinon, first produced commercially in 1952,1 is still a widely used organophosphate pesticide. It is used to control a wide variety of suckling and leaf eating insects. It is used on rice, fruit trees, sugar cane, corn, tobacco, potatoes, as well as horticultural plants. It is also an ingredient in pet strips and a domestic pest control agent. Finally, it is used to control ectoparasites on livestock. Therefore, there is the potential for exposure to a large proportion of the population, with the most heavily exposed being those working in agriculture2–4 and the chemical industry.5 Although there is a tendency for the use of organophosphate pesticides in Europe to be reduced, pesticide abuse is a serious problem in Crete. Many cases of pesticide poisonings, due to acute and subacute exposure, have been reported.6–9 Diazinon is one of the most popular organophosphates in Crete, used to control insects in cultivations, livestock and in houses. As a result, quite a large number of people, not only farmers, are exposed to it.

Diazinon exerts its action by the phosphorylation of acetyl cholinesterase, resulting in inhibition of the enzyme, acetylcholine accumulation and altered cholinergic neurotransmission.10 Although diazinon itself is not a potent enzyme inhibitor, in animals and humans it is metabolized by cytochrome P450,11 to form diazoxon, a compound that is a very strong enzyme inhibitor.12 The acute toxicity of diazinon is not very high but chronic exposure to the compound, even at very low levels, may cause a series of adverse effects.13,14 Several studies have indicated that it may be mutagenic, or cause developmental toxicity in various animal species.15 All the above indicate the necessity of an easy, non-invasive method of assessing chronic exposure to the pesticide.

Hair, unlike short-term indicators, e.g., body fluids, retains an incorporated substance for long periods since there is no active metabolism/excretion to remove it once it is deposited. Segmental hair analysis has been successfully used to assess chronic exposure to various chemicals, such as
drugs of abuse, heavy metals and so on. Very recently it has been shown that several environmental pollutants, such as organochlorine pesticides and PCBs, can be detected in hair. A study has indicated that methomyl may also be detected in hair. The aim of the present work is to design and evaluate a simple method of extraction and analysis of diazinon from hair, which will assist in determining whether diazinon does accumulate in hair in measurable quantities. Rabbits were used as the animal model that would show whether diazinon accumulates in hair.

Materials and methods

Animals
Three groups of white rabbits of the New Zealand variety were used for the experiment. Each group consisted of five rabbits. One was used as a control and the others received two different dosages of the pesticide in their water. The high dosage group received approximately 15 mg/kg per day and the low dosage group received 8 mg/kg per day, contained in about 100 mL of water, for a period of 4 months. Before commencing the experiment they were treated for internal and external parasites and they received antihaemorrhagic vaccine. They were kept in a 12-hour dark/light cycle and they were fed with rabbit pellets ad libitum. The experimental protocol was approved by the Veterinary Administration Office of Heraklion, Ministry of Agriculture and conformed to the National and EU directions for the care and treatment of laboratory animals.

Sample collection
At the end of the dosing period, hair was removed from the back of the rabbits, rinsed in methanol, dried and stored at room temperature until analysis. Blood was collected from the ear veins into vacuum tubes using 1-mL syringes and, after centrifugation, the serum was analysed immediately and then stored at −20°C.

Reagents
All solvents used were of high-performance liquid chromatography (HPLC) grade and were supplied by Merck (New Jersey, USA). The pesticides diazinon and fenthion were of analytical grade and a generous donation of Ciba Geigy Hellas S.A. (Anthousa, 153 44 Greece).

Serum cholinesterase measurements
The activity of acetyl cholinesterase was determined at the beginning and at the end of the dosing period, using a kit from Roche (Basil, Switzerland). Blood samples were centrifuged immediately in order to obtain serum and the instructions of the kit were followed precisely. The activity was determined spectrophotometrically at 405 nm (Jasco 7800 Model, UV/Visible spectrophotometer, Tokyo, Japan). Reference values for humans range between 5300 and 12 900 U/I. Reference values for the rabbits ranged between 404 and 500 U/I. Absorbance measurements were performed against distilled water. Student’s t-test was applied for the statistical analysis of the results. The results were considered as significantly different when the P-value was smaller than 0.05.

Diazinon extraction from hair
The efficiency of the extraction methods was tested on fortified hair. Three methods were applied for the extraction of diazinon from hair. Acidic and basic hydrolysis proved to be very harsh for the pesticide and as a result it could not be detected in any of the chromatograms. Methanolic extraction of the pesticide from hair proved to be the most suitable.

Initially, 100 mg of hair was weighed and pulverized in a ball mill homogenizer. The powder was transferred in a test tube with 2 mL of methanol together with the internal standard (2 ng/mg final concentration fenthion) and was incubated at 37°C overnight. The supernatant was transferred to a clean test tube and methanol was evaporated to dryness under a gentle nitrogen stream. The residue was resuspended in 2 mL of HPLC grade water and liquid–liquid extraction followed with 3 mL of ethyl acetate twice. The organic phase was transferred to a clean test tube and evaporated to dryness under nitrogen. The residue was resuspended in 50 μL of ethyl acetate and analysed by gas chromatography–nitrogen phosphorus detector (GC-NPD) and gas chromatography–mass spectrometry (GC-MS).

GC-NPD analysis
One microlitre of the test solution was injected in the splitless mode, in a Carlo Erba GC 6000 Vega Series 2 instrument (Milan, Italy), which was coupled to an NPD and was equipped with a DB-1 30 m × 0.32 mm capillary column (J&W Scientific, Folsom, USA). The conditions of analysis were as follows. Column temperature started at 180°C, where it remained for 3 min. Then it increased up to 230°C at a rate of 5°C/min, steady for 5 min and finally it increased up to 265°C, at a rate of 10°C/min, where it remained for 15 min. The injector temperature was set at 250°C and the detector temperature was set at 290°C. Helium with a flow rate of 2.1 mL/min was used as a carrier gas. Under
these conditions, diazinon eluted at time $t = 25.49$ min, and fenthion eluted at time $t = 33.57$ min. The diazinon hair concentrations were compared by Student's $t$-test. The difference was significant when the $P$-value was smaller than 0.05.

**GC-MS analysis**

Electron ionization mass spectrometric analysis of hair extracts was performed on a Finnigan Mat GCQ system (Austin, USA) equipped with a AT-5 MS (30 m $\times$ 0.25 mm $\times$ 0.25 $\mu$m) capillary column supplied by Alltech (Deerfield, USA). Pure helium with a velocity of 20 cm/s was used as a carrier gas. One microlitre of the solution was injected into the system in the splitless mode and was analysed under the following conditions: the column temperature was initially held at 180°C for 3 min, raised to 230°C at 10°C/min, held for 2 min, and was finally raised to 300°C, at 20°C/min, where it remained stable for 2 min. The injector temperature was 200°C. The transfer line temperature was set at 275°C. The mass spectrometer acquisition parameters were: ion source 200°C, electron impact ionization at 70 eV and electron multiplier voltage of 1200 V. The mass spectrometer was operated at the selected ion-monitoring mode and programmed for the detection of $m/z$ 179 and 304 for diazinon, and 278 for fenthion. Under these conditions diazinon eluted at time $t = 16.14$ min, and fenthion which was used as the internal standard eluted at time $t = 18.63$ min.

**Recovery experiments**

The recovery evaluation of the extraction method was performed on spiked hair. It was experimentally determined at two concentration levels. The pesticide was added to 100 mg of blank hair, to the final concentrations of 0.6 and 1 ng/mg hair. In the first case the pesticide and the internal standard were added at the beginning of the extraction procedure, while in the second the hair was treated in an identical manner to the first but the analytes were added in the last step. The extracts were analysed by GC-NPD analysis. The ratio of the area of the analyte to that of the internal standard was calculated for each concentration point of the two groups. Then the ratio given for the first group was divided by that of the corresponding concentration given by the second group in order to determine the recovery ratio, which was then multiplied by 100 in order to find the percentage recovery.

Quantification of the pesticide was done by GC-NPD. GC-MS analysis was only performed on selected samples for confirmation. An eight-level calibration curve was prepared using methanolic standard solutions in order to evaluate the method. The curve was linear between the concentrations 0.1 and 1 ng/mg (Figure 1).

**Results**

The results depicted in Table 1 show that acetyl cholinesterase activity was $468 \pm 118$ I/U in the control group, $401 \pm 48$ I/U in the low-dose treatment group and $245 \pm 68$ I/U in the high-dose treatment group. The activity of the enzyme was significantly decreased in both treated groups.

Hair analysis was performed using GC-NPD (Table 2, Figure 2) for quantification and GC-MS for confirmation (Figure 3). Each sample was measured in triplicate. Blank samples from the untreated animals were run to ensure lack of interference.

The recovery of the target compounds with the employed method was estimated as 69%. The standard curve prepared for the sample quantification was linear between the concentrations 0.1 and

<table>
<thead>
<tr>
<th>Group</th>
<th>Rabbit</th>
<th>Blood acetyl cholinesterase I/U</th>
<th>Group mean values</th>
<th>t-test</th>
<th>P-value</th>
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<td>Untreated</td>
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<td>468 $\pm$ 118</td>
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<td>$445 \pm 5$</td>
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<td>4</td>
<td>$404 \pm 3$</td>
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<tr>
<td></td>
<td>5</td>
<td>$500 \pm 10$</td>
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<tr>
<td>Low dose</td>
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<td>$401 \pm 48$</td>
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a Significance levels estimated for high- and low-dose groups compared with the untreated group; $n = 5$. The values given are the mean of three measurements. The group values are the mean of group measurements.
Discussion

Diazinon exerts its pesticidal action mainly through the inhibition of the enzyme acetylcholinesterase. Blood acetylcholinesterase activity measurements were an indication of the intoxication of the treated animals. As indicated by the results, the activity of the enzyme in both treatment groups was significantly decreased and the decrease was related to the administered dose. There is some indication that apart from the effects on the target enzyme, chronic exposure to low levels of diazinon may result in a series of adverse effects such as reduced bone formation.\textsuperscript{25} As a result a method that is suitable to measure chronic exposure to the pesticide is necessary.

In recent years hair has been a matrix that is extensively studied in order to assess chronic exposure to various chemicals, the best studied being drugs of abuse. A literature research has revealed that apart from a study about methomyl disposition in rabbit hair performed in our lab,\textsuperscript{23} the disposition of organophosphate or carbamate pesticides in hair of subjects systemically exposed has not been studied at all. On the other hand a lot of work has been done to measure the concentration of heavy metals like mercury\textsuperscript{26} and pesticides such as lindane\textsuperscript{27} or environmental pollutants like PCBs\textsuperscript{22} in hair.

The main aim of the present work was to determine whether diazinon can be detected in the hair after chronic exposure and if there is a relationship between the diazinon dose administered for a certain time period and the pesticide concentration measured in hair. Since a literature search revealed no analytical method to measure the pesticide in hair, it was necessary to establish and evaluate a simple method by which diazinon may be extracted from hair and measured reliably. Hair samples were removed from the back of the rabbits and analysed as a total. Three methods were applied for the extraction of diazinon in hair. The pesticide proved to be unstable in acidic and alkaline hair hydrolysis. As a result no peak was detected in the chromatograms. Also prolonged incubation at 40°C or more decreased the recovery of the analytes of interest. The described method of extraction proved to be easy and reliable enough, giving a mean recovery of 69%. It takes advantage of the great solubility of diazinon in organic solvents such as methanol and ethylacetate. Methanol is very efficient to extract the compounds of interest once present in solution.

Quantitative analysis was facilitated by the use of GC-NPD, a very selective detector of compounds that contain nitrogen and phosphorus; as a result the chromatograms were almost free of interference peaks.

As can be seen from the results, diazinon can be detected in hair samples of the exposed animals.
Also the results indicate that the concentration in hair is related to the administered dose. In this experiment white animals were used. The generally low concentrations of the trapped pesticide in hair could be attributed to the absence of melanin. Melanin of the hair is known to be a binding site for the trapped drugs. Therefore, it would be very interesting to examine the effect that hair colour may exert on the concentration of the measured compound, since previous studies with other compounds have indicated that the colour of hair influences the amount of the bound drug.  
Overall, our report supports the idea of using hair testing as a marker for the exposure history of an individual to organophosphate pesticides. Of course various issues such as the effect of hair growth rate or the effect of hair colour on the concentration of the drug in hair must be clarified before quantitative assessment of exposure to pesticides using hair can be discussed.

References

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