Chapter 24

Assessing Chronic Exposure to Anticholinesterase Pesticides by Hair Analysis

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Abstract. The present paper explores the possibility of using hair as a suitable biomarker of chronic exposure to anticholinesterase pesticides. Research results already available confirm that hair is a suitable biomarker of past exposure to drugs and pharmaceuticals. Recent experimental evidence suggests that hair is a suitable biomarker for the assessment of chronic exposure to organophosphate and carbamate pesticides. Animal studies have indicated that hair concentrations of the pesticide diazinon are dose dependent. Hair has been used as biomarker of chronic and recent exposure to anticholinesterase pesticides in an epidemiological study conducted in Crete. Experimental data confirmed the presence of organophosphates in hair samples of the rural population examined.

Keywords. Organophosphate pesticides, hair, biomarker, gas chromatography-mass spectrometry, chronic exposure

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Introduction

Toxicological analysis of non-conventional biological samples (e.g. hair, saliva, sweat, and sperm) has significant value and many applications in several areas of medical, forensic and environmental science. These samples may provide important additional information and possess certain advantages (e.g. sampling is time-efficient, practical, cost-effective, non-invasive and a second sample giving similar information to the first can be easily obtained) over the conventional biological samples. Among the non-conventional biological samples, hair can provide the most vital information, with sectional hair analysis being the most widely used application [1].

The main advantage of hair is that it retains trapped information for prolonged periods of time. Analyte stability in hair has been demonstrated in 4000 years old mummies, in which small amounts of cocaine metabolites were found [2]. This is attributed to the absorption and trapping mechanism that exists in the hair, taking place during keratinisation of the newly formed cells.

Several studies have provided contradicting evidence on the dose-response relationship between chemical ingestion and chemical titres in the hair. Despite the concerns expressed by the scientific community with respect to the role of hair testing, it has already been applied in forensic investigations, historical research, autopsy, adoption cases, exclusion of evidence, serial criminal cases, rape cases, doping control, as well as other scientific and/or legal cases [3]. Another possible application would be in clinical practice. Sectional hair testing may be utilized to check compliance to therapy regime for people under long-term treatment for several diseases [4].

Hair analysis has been successfully used to assess chronic exposure to various chemicals like drugs of abuse, medicines and heavy metals. Recently, analysts give their attention to the possibility of using hair analysis for the assessment of exposure to organic pollutants. The most studied compounds are organochlorine pollutants, like pesticides, dioxins and PCBs [5-13] and currently used pesticides belonging to the families of anticholinesterase agents like organophosphates (e.g. diazinon, malathion) [14-16] and carbamates (e.g. methomyl, carbaryl) [17-18]

Hair analysis is feasible due to the special characteristics of hair, and the most basic ones are described below.

Although hair might appear as a simple and homogeneous structure to a non specialized observer, it is in fact a very complex part of human anatomy. Hair consists of long shafts created of closely packed cells that emerge from the follicles. The mean diameter of a single hair shaft ranges from 15-120 μm, in humans, depending on the type of hair and the body area that each follicle is located. Hair is rich in keratins, a family of proteins with a high content of sulfur. Inside the shaft, keratin forms long fibers linked to each other by sulfur bridges and other types of bonds between keratin and other proteins, creating a very stable structure

The hair could be described as cross linked polymer containing a large number of chemical functional groups, capable of trapping small molecules. Human hair consists of 65-95% proteins, 15-35% water and 1-9% lipids. Lipids originate from sebum and the secretions of apocrine glands and consist of free fatty acids, mono and di-glycerides, waxes carbohydrates and aliphatic alcohols. Human hair is rich in the aminoacids threonine, aspartic acid, glutamic acid, lysine, cystine and tyrosine [19].

Substances in the blood-circulating system, which enter the hair via the follicle, are trapped and retained in specific parts of the hair. Sweat and sebaceous glands also play a basic role in the process of drug deposition in hair. Water-soluble compounds excreted into sweat and sebum from the skin may also be incorporated in the hair. The removal of drugs depends upon several variables, such as gels or solutions used to wash or treat the hair [20].

Hair analysis for the assessment of exposure to Anticholinesterase pesticides

Pesticides are substances that are used to prevent, destroy, repel or mitigate any pest ranging from insects, animals and weeds to microorganisms such as fungi, moulds, bacteria and viruses or other organisms that compete with humans for food, destroy property, spread disease, or are considered a nuisance [21-22].

Workers employed in the manufacture and application of pesticides is the most highly exposed group [17]. While acute exposure to pesticides usually leads to intoxication with well defined and studied symptoms and only concerns a small part of the population, the interest of public health officials is focused in understanding and recording the effects of low level long term exposure to pesticides. This is because pesticides are widely released into environment. Consequently, exposure of the general population at some level to several different pesticide residues is almost inevitable [23].

In vivo studies in animals

Recently, researchers have demonstrated that organophosphorus pesticides and environmental pollutants (DDTs and HCHs) can be detected in hair [24-26]. The disposition of diazinon in the hair of experimental animals that were exposed to the pesticide through their drinking water was studied in our laboratory. [27-28].

Rats and rabbits were exposed to the pesticide diazinon through their drinking water. Both rats and rabbits were divided to three groups. One served as the control group and the two others received the pesticide in their drinking water at two dose levels. The rats were exposed to two levels of the pesticide, a low one of 2.7 mg/kg/day and a high one of 5.5 mg/kg day in their drinking water for 45 days. The rabbits were exposed to 7 mg/kg/day (low dose) and 15 mg kg/day (high dose) through their drinking water for four months. At the beginning and at the end of the dosing period, as soon as the hair regained its original length, it was removed from the back of the experimental animals.

A sensitive and selective method for analyzing organophosphate pesticides like diazinon, fenthion and methyl parathion in hair was developed. This method includes preparation of the sample by homogenization of the hair, methanolic extraction followed by liquid-liquid extraction with ethyl acetate and analysis by gas chromatography- mass spectrometry or gas chromatography coupled to nitrogen phosphorus detector. Analysis of the samples of both animals revealed that the concentration of the pesticide detected in hair was dose dependent [27-28].

Table 1: Diazinon concentration in the hair of the exposed rats and rabbits.

Animal	Group	Group mean concentration (ng/mg) of hair ± st.dev.	T-test p value
	Control group	0	
rat	Low dose (2.7 mg/kg)	0.24 ± 0.01	<0.001
	High dose (5.5 mg/kg)	0.53 ± 0.05	
	Control group	0	
rabbit	Low Dose (7 mg/kg)	0.17 ± 0.05	0.023
	High dose (15 mg/kg)	0.23 ± 0.02	

†Significance levels estimated between the high and low dose groups. n=5

In vitro studies using human hair.

The effects of parameters such as colour of hair, concentration of pesticide and time of hair exposure to the pesticide diazinon were studied *in vitro* using human hair.

Three experiments were conducted in order to study the effect of the aforementioned parameters.

In order to study the effect of time of exposure of the hair sample to the pesticide, a 100 mg pesticide free sample of brown hair was immersed in an aqueous solution of 0.01 mg/ml diazinon for 1 and 4 h, at ambient temperature. At the end of each incubation period, the hair was removed from the solution, washed for two min in methanol in order to remove the loosely bound pesticide, and analysed by GC-MS.

Results (Table 2) indicated that the measured hair concentration of diazinon was time dependent.

Table 2: Measured concentration of diazinon in hair, following incubation of the sample in a 0.01 mg/ml aqueous solution of the pesticide.

Concentration of exposure medium (mg/ml)	Time of exposure (h)	Concentration of diazinon in hair (μg/mg)
0.01	1	0.46
0.01	4	2.22

In order to study the effect of the pesticide concentration in the exposure medium, samples consisting of 100 mg of natural brown hair were incubated in aqueous solutions of diazinon at various concentrations (0.01 mg/ml, 0.1 mg/ml και 1 mg/ml) for 1 h. At the end of the incubation period the samples were removed from the solutions simultaneously, processed and analyzed by GC-MS.

It is easily observed that the concentration of the pesticide in the exposure medium plays a crucial role in determining the levels of the pesticide absorbed by hair. Presumably saturation of the available binding sites in the hair would account for a leveling of the measured pesticide concentration.

Previous studied have indicated that hair colour is a crucial parameter that determines the amount of chemicals that may bind to hair, especially if absorption of the substance in hair occurs through the bloodstream, during the keratinisation step of the

hair shaft More specifically it has been found that the concentration of many substances measured in hair is proportional to the melanin content of the hair shaft [29]. In order to study the effect of colour on the detected diazinon concentration, in the case of external contamination of hair with diazinon, human hair samples (100 mg) of different colours were incubated in an aqueous solution of diazinon of 0.1 mg/ml for 1 h. At the end of the incubation period hair samples were removed from the incubation media, processed and analysed by GC-MS. The results of the experiment are depicted in table 4.

When diazinon is absorbed through external contamination of the intact hair shaft from the environment and not through the blood stream during the keratinazation step of the hair shaft, melanin content does not seem to play such an important role. The

Table 3: Measured concentration of diazinon in hair following 1 h incubation in diazinon aqueous solutions of different concentrations.

Diazinon concentration of exposure medium (mg/ml)	Time of exposure (h))	Concentration of diazinon in hair (μg/mg))
0.01	1	0.10
0.1	1	1.59
1	1	3.99

Table 4: Measured diazinon concentration (μg/mg) in hair samples of different colours incubated in 0.1 mg/ml aqueous solution of diazinon for 1 h.

Sample	Colour	Diazinon detected concentration (μg/mg)
1	Brown (dyed)	3.3
2	Light brown	2.2
3	Blonde	1.3
4	Black	0.9

results of the conducted experiment show differences in the measured hair diazinon concentration, but these do seem to relate so much to the melanin content of the hair shaft. This could be exposed by the fact that melanin is not directly exposed to the environment. On the contrary, the hair shaft is enclosed by a membrane that protects the cells that carry the melanosomes. The destruction of the membrane is necessary before chemical substances gain access to the melanosomes and the melanin binding sites.

Hence it seems that other factors play a role in the amount of pesticide binding to intact hair through passive exposure from the environment. These parameters would be the state of the membrane, as indicated by the fact that dyed hair absorbed more pesticide than the hair with intact membrane. Other parameters could be the lipid content of the hair. Hair with higher lipid content offer more binding sites to diazinon than hair with lower lipid content.

Finally another factor that could account for the amount of diazinon bound in hair is the diameter of the hair shaft. Hair shaft with smaller diameter offer more binding sites to diazinon per equal weight of sample than those with bigger diameter.

As a summary we should note that when measuring the concentration of a pesticide in hair several factors must be studied before relating the measured pesticide in hair to the exposure of the organism.

Population study in humans

Pesticide exposure of rural and urban population in the island of Crete was assessed using hair analysis.

A total of 463 and 70 head hair samples from rural (South Crete) and urban population (Heraklion city, Crete) were collected and analyzed. Approximately 500 mg of hair was cut from the root, at the back of the head and used for the analysis. Hair samples were analyzed for the currently used organophosphate pesticides (diazinon, methyl parathion, fenthion, malathion, dimethoate and chloropyrifos). Also they were analysed for the hexachlorocyclohexane isomers (HCHs: lindane, a HCH, HCB) and the dichloro-diphenyl trichloroethane isomers (DDTs: op-DDE, pp-DDE, opDDD, pp-DDD, op-DDT and pp-DDT).

The length of the samples from male participants varied from 3 up to 6 cm, corresponding up to 6 months of pesticide exposure, while that of the female participants varied from 8 up to 32 cm, corresponding up to 32 months of pesticide exposure [26, 30].

Organophosphate extraction

The first step of the sample preparation procedure was the removal of the external contamination from the hair matrix. Hair was washed twice in water and methanol for two minutes and dried in the oven at 38^{o}C. Subsequently 200 mg of hair was weighed out and pulverized in a ball mill homogeniser. Pulverisation of the hair before methanolic extraction was necessary for optimal recovery of the target analytes.

The powder was transferred in a test-tube with 2 ml of methanol and was incubated at 37^{o}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 combined organic phases were transferred to a clean test-tube and evaporated to dryness under nitrogen. The residue was resuspended in 50 µl of 100 ng/ml 1,2,3,4-tetrachloronaphthalene (TCN) solution in hexane (external standard) and analysed by gas chromatography – mass spectrometry (GC-MS).

Results and Discussion

In the present study we assessed the present and past exposure to four currently used pesticides (diazinon, methyl parathion, malathion, fenthion), as well as to two banned ones that are still found as environmental pollutants and their metabolites (HCHs and DDTs) using hair analysis.

The concentration of the detected HCHs was higher in the hair of the rural population, compared to the urban one, but they were detected in both population groups.DDTs were also detected in both groups at very similar concentration levels.

No OPs were detected in the hair of urban population. Diazinon was detected in 2.8% of the hair samples of rural population with concentrations ranging from 2.5 to 5.8 pg/mg, malathion in 1.5% of the samples (5.1-8.4 pg/mg) and chloropyrifos in 2.4 % of the samples (5.0-11.3 pg/mg). Methyl parathion, fenthion and dimethoate were not detected in any hair sample (Figure 1).

It was observed that the large majority of the examined samples was negative for all of the examined analytes. This is a good indication that these pesticides either degrade fast in the environment and in the organism, or that there was insignificant exposure due to strict observation of the safety rules (respiratory masks, gloves, special clothing) during the manipulation or application of the pesticides. Also the concentrations detected in hair were close or below the quantitation limit achieved presently. For this reason, our next step is to focus to the determination of specific and non specific metabolites (dialkyl phosphates) of organophosphates pesticides in hair [31]

A literature search revealed that not many published studies exist on the setection of pesticides in hair. In report by Liu and Pleil [14] it is stated that three OP pesticides were detected in hair, diazinon at a concentration of 20ng/g, chlorpyriphos at a concentration range of 33-700 ng/g and Malathion at concentration range of 7-24 ng/g.

Ostrea and co-workers analyzed cord blood, infant hair and meconium samples simultaneously to determine the most sensitive matrix to detect antenatal pesticide exposure [32]. In this comparison analysis of infant hair, cord blood and meconium the authors reached the conclusion that meconium was the best matrix for this purpose.

The pesticides under investigation were propoxur, diazinon, lindane, transfluthrin, malathion, chlorpyriphos, bioallethrin, pretilachlor, DDT, cyfluthrin and cypermethrin. Eight of these pesticides were detected in meconium with a frequency between 0.2 % for diazinon to 23.8 % for propoxur. Cord blood and infant hair were positive, each for a single pesticide, propoxur and chloropyrifos respectively.

All the above evidence suggests that among other uses, hair analysis despite the uncertainties that exist in the interpretation of the results in relation to the observed concentrations, gives valuable information about exposure to pesticides.

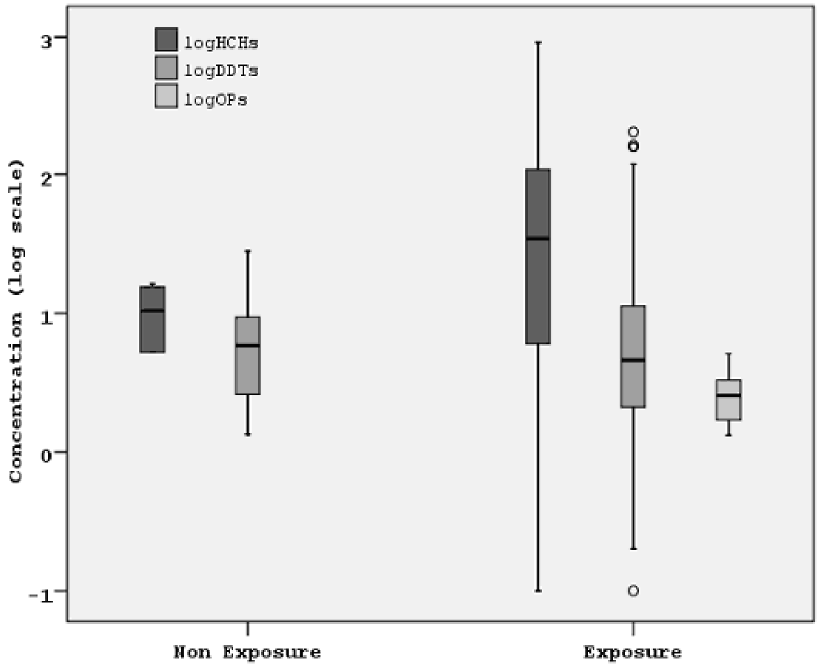

Figure 1: Concentration range of the analyzed compounds in the hair of urban (non exposed) and rural (exposed) population of Crete

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