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Porphyrins as Biomarkers of Methylmercury and PCB Exposure in Experimental Quail

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Chemicals such as heavy metals and polyhalogenated hydrocarbons have a high capacity to interfere with the enzymatic processes responsible for haem biosynthesis. These compounds can produce accumulation in tissues and organs and increased elimination of porphyrins in excreta (Andrew *et al,* 1990). The development of fast and easy analytical methods and the wide variety of biological media in which porphyrins can be detected have suggested their use as biomarkers of environmental pollution (Akins *et al,* 1993; De Matteis and Lim 1994). The analysis of porphyrins in the excreta is of special interest because it enables non-destructive monitoring of wild animals in the assessment of threatened or endangered species (Fossi *et al,* 1994).

Methylmercury and PCBs are ubiquitous global pollutants and there is evidence they accumulate in terminal consumers, particularly those belonging to marine trophic chain (Renzoni *et al,* 1986; Yamashita *et al,* 1993). There have been some reports on methylmercury-induced (e.g. Woods *et al,* 1991; Bowers *et al,,* 1992; Miller and Woods 1993) and PCB-induced porphyria (e.g. Vos and Pennings, 1971; Miranda *et al,* 1987; Elliot *et al,* 1990; Miranda *et al,* 1992) but little data on their combined effect.

In order to investigate the quality of porphyrins as biomarkers we performed an experiment in which Japanese quail were fed a diet containing methylmercury and polychlorobyphenyls (PCBs as Arochlor 1260) individually or combined in different ratios. The present study aims to provide preliminary data on liver and fecal levels of porphyrins in response to methylmercury and PCB administration, and on whether the indicator is sensitive to synergism or antagonism between the two compounds, administered simultaneously.

MATERIALS AND METHODS

Forty adult male Japanese quail (*Coturnix coturnix japonica*) were acclimatized and then divided into seven groups: a group of ten quails was kept as controls and the other 6 groups, each of five quail, were fed diets containing: 2.5 mg/kg of mercury as methylmercury (ME2.5), 25 mg/kg of mercury as methylmercury

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(ME25), 10 mg/kg Arochlor® 1260 (PCB10), 100 mg/kg Arochlor® 1260 (PCB 100), 2.5 mg/kg of mercury as methylmercury plus 10 mg/kg Arochlor® 1260 (ME2.5+PCB10), 25 mg/kg of mercury as methylmercury plus 10 mg/kg Arochlor® 1260 (MB25+PCB100). These diets were suspended after 3 weeks. The birds were sacrified and the livers processed immediately for porphyrin determination. Excreta were pooled during the last three days of treatment in order to have enough, and more homogeneous material, then freeze-dried and homogenized for porphyrin analysis.

Porphyrins in the liver were extracted by the method of De Matteis and Lim (1993). About 300 mg of liver was taken and homogenized 1:10 in distilled water in a Turrax homogenizer. 0.2 ml of homogenate was then transferred to glass tubes to which a mixture of a methanol/perchloric acid (50:50)(1.6 ml) was added. After Vortex mixing the samples were kept in the dark for 10 minutes and then centrifuged for 5 minutes at low speed. The porphyrin extract in the upper layer was then used for spectrofluorimetric determination.

The method of Lockwood *et al*, (1985) was used to extract porphyrins from excreta: 1 ml of 5N HCl was placed in a graduated centrifuge tube containing an accurately weighed excreta sample (100 mg) and Vortex-mixed. Diethyl ether (3ml) was added and thoroughly mixed to produce an emulsion, followed by water (3ml) and further mixing. The mixture was then centrifuged at 700 x g for 10 minutes. The lower layer of aqueous acid containing the porphyrin was taken for fluorimetric determination.

The method of Grandchamp *et al*, (1980) was used for the quantitative determination of porphyrins. This fluorimetric procedure is used to determine the

Table 1. Copro, uro, proto and total porphyrins in liver and faeces (pmol/g fresh liver tissue weight and pmol/g of lyophilized excreta weight) (arithmetic mean ± standard deviation, n=5 for each group) and multiple range analysis (MR) (different letters denote a statistically significant difference for $p<0.05$)

LIVER	COPRO	MR	URO	MR	PROTO	MR	TOTAL	MR
CONTROLS	17±14	A	187±29	AB	477±89	A	680±110	A
ME2.5	15±12	A	252±120	BC	453±131	A	721±32	A
ME25	158±11	B	170±45	A	749±209	BC	1078±300	B
PCB10	141± 91	B	215±26	ABC	852±123	CD	1210±186	B
PCB100	89±74	AB	274±30	C	785±161	BC	1150±215	B
ME2.5+PCB10	54±64	AB	217±40	ABC	877±191	AB	877±191	AB
ME25+PCB100	277±115	C	265±56	C	1052±218	D	1595±329	B

EXCRETA	COPRO	MR	URO	MR	PROTO	MR	TOTAL	MR
CONTROLS	766±268	A	178±39	A	848±327	A	1650±109	A
ME2.5	760±258	A	205±126	A	769±200	A	1730±553	A
ME25	2724±1682	B	675±402	C	2137±162	B	5531±3051	B
PCB10	1080±1001	A	315±270	ABC	884±162	A	2323±1411	A
PCB100	2014±739	AB	658±280	C	1808±254	AB	4481±1132	B
ME2.5+PCB10	752±97	A	245±56	AB	1067±258	A	2050±380	A
ME25+PCB100	5757±1971	C	589±437	CD	4227±1672	C	7720±5190	B

percentages and concentrations of uroporphyrin, coproporphyrin and protoporphyrin in a mixture of porphyrins in the nanomolar range. The procedure is based on the different excitation/emission wavelength couples of each porphyrin (uroporphyrin: 405-595nm; coproporphyrin: 400-595nm; protoporphyrin: 410-605nm). By recording the fluorescence emission of the mixture at the three different pairs of values, three linear functions representing the concentrations of each porphyrin are obtained.
The porphyrin samples were placed in a micro-cuvette and measured using a Perkin Elmer LS-50B spectra-fluorimeter. The results were processed by summary statistics and the multiple range test (Box *et al,,* 1987) using STATGRAPHICS software (Statistical Graphics Corporation).

RESULTS AND DISCUSSION

The results of the porphyrin determination are reported in Table 1. For each treatment group, total, copro, uro and protoporphyrins are reported together with standard deviations in brackets and the results of the ANOVA multiple range test. Total porphyrins were considerably increased in the ME25, PCB100 and ME25+PCB1000 group with respect to controls. Porphyrin levels were higher in liver of PCB10 group than in controls. Cumulating results of all the treatments, total porphyrins in liver were positevely correlated with total porphyrins in excreta (Table 2; Figure 2).
Coproporphyrin levels varied widely in relation to treatment. Significant differences with respect to controls were found in liver for the groups PCB 10, ME25 and ME25+PCB100 and in excreta in groups ME25 and ME25+PCB100. Uroporphyrin levels were higher in the liver of groups PCB 100 and Me25+PCB100 and in the excreta of groups Me25 PCB100 and ME25+PCB100. Protoporphyrins level showed significant differences with respect to controls in the groups PCB 10, PCB 100, ME25 and ME25+PCB100 for liver and in the last two also for excreta.
Percentage differences with respect to controls are reported in Figure 1. Total porphyrins show a higher percentage variation in excreta than in liver particularly for high-dose groups, singly and combined. The combined group ME25+PCB100 showed an almost additive effect in liver and excreta. Porphyria in the liver is mainly due to the presence of coproporphyrin that was dramatically induced by

Table 2. Correlation matrix (correlation coefficient and p value below) between liver porphyrins (L) and porphyrins in the excreta (E) of quails (all treatments + controls; n= 40) .

	L COPRO	L URO	L PROTO	L TOTAL
E COPRO	0.7700 *	0.0532	0.6855*	0.7443*
E URO	0.3416	0.0575	0.3424	0.3536
E PROTO	0.7007 *	0.3122	0.6848	0.7382
E TOTAL	0.5662 *	0.0998	0.5866 *	0.6004 *

p < 0.001

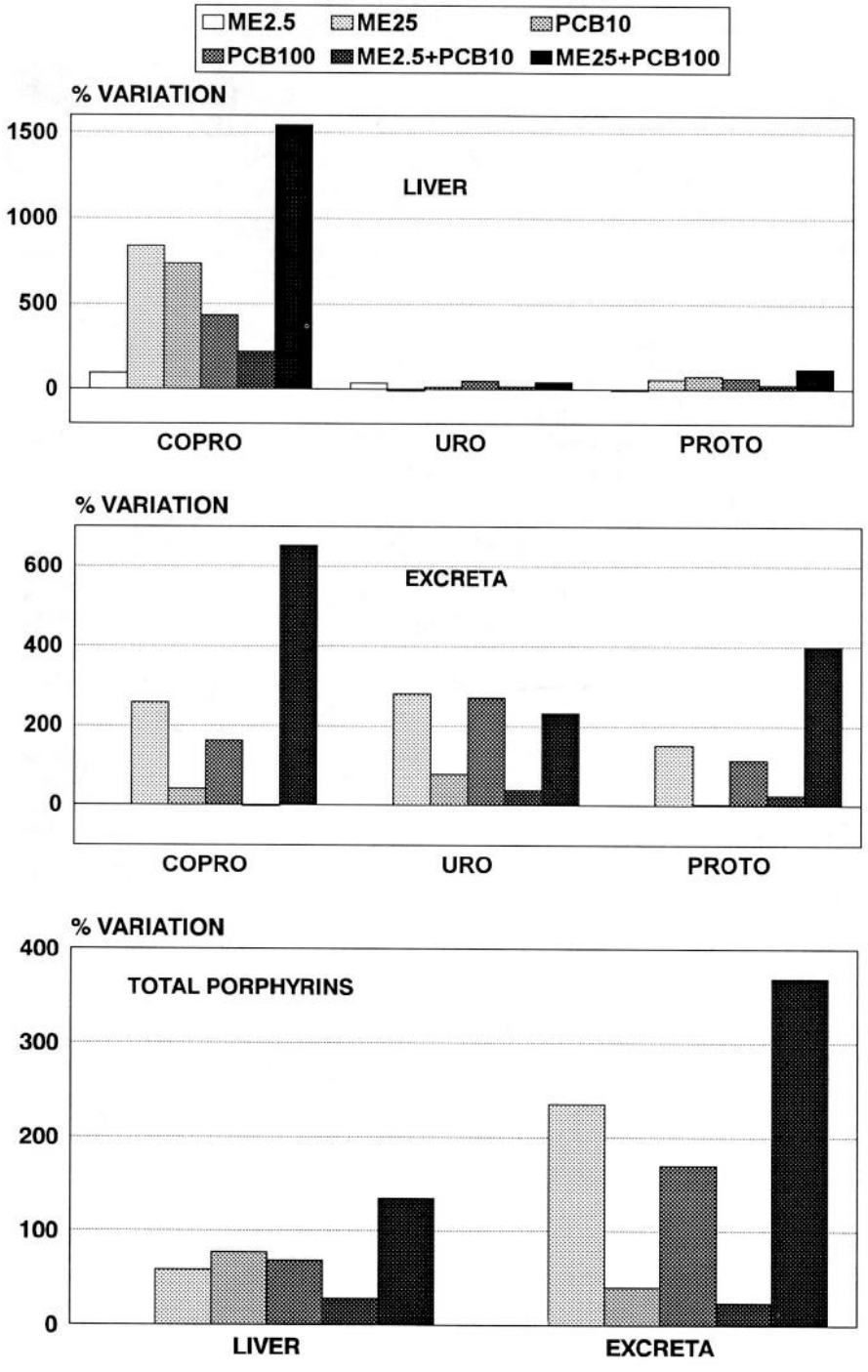

Figure 1. Average percentage variation of porphyrins in treated groups with respect to controls (0 line) in the liver and excreta.

almost all treatments with percentage variations from 100 to 1500%.
In the excreta all the porphyrins analyzed showed significant variations. PCB10 treatment had more effect on protoporphyrin levels than PCB100 treatment. Combined treatments showed a non-additive but rather antagonistic effect in the low-dose group (ME2.5+PCB10) conversely, in the mixed high-dose group (ME25+PCB100) the effect was more than additive (except for uroporphyrin), with control values increasing from 215 to 620%.
Correlations between porphyrins in excreta and liver are reported in Table 2. There was a highly significant inter-correlation ($p<0.0001$) between total, copro and protoporphyrins of liver and excreta. Uroporphyrins were not significantly correlated with the others.

From this data it emerges that:
a) porphyrin levels in groups treated with low doses of methylmercury and PCBs were difficult to distinguish from those of controls; b) porphyrin levels in high-dose groups were nearly always significantly different from controls; c) in some cases mixed treatments showed a more than additive, even synergic, effect.
Effects of PCBs on hepatic porphyria are reported by other authors (Vos *et al,,* 1971, Bowers *et al,,* 1992). Elliot *et al,* (1990) found significant porphyrin accumulation in liver of japanese quail chronically dosed with 2,3,3',4,4'-PCB with a pattern similar to that found in the present study.
A previous study on the effect of methylmercury (Woods *et al,* 1991) reported that the urinary excretion of porphyrins in rats treated with 5 and 10 ppm of methylmercury increased during the first 10 weeks of treatment. A similar phenomenon presumably occurs in quails, with a progressive increase in liver and urinary porphyrins for treatment periods exceeding 3 weeks (as in our experiment).
In general it seems that porphyria depends on both daily intake of chemicals and to period of exposure (De Matteis and Lim, 1993). Low doses such as those used in the present experiment could give a very sensitive response over a longer period. This is an important aspect for the validation of porphyrins in field studies with organisms that may be exposed to chemicals inducing porphyria for the period of their lives.
Nearly all the variations observed in the high dose treatments were statistically significant, the percentage increases reaching very high levels (+1500%) with respect to controls for mixed treatments. The highest concentrations of total porphyrins were found in excreta, individual and total porphyrins showing great sensitivity of response.

The preliminary data emerging from the present study suggests that certain porphyrins and total porphyrins can be used as biomarkers of exposure to methylmercury and PCBs. The maximum sensitivity of these biomarkers seems for the moment to be linked to high inputs of the compounds and/or long exposure time. The simultaneous presence of methylmercury and PCBs in the diet either depresses or does not modify porphyrin level at low doses but has an additive or synergistic effect at high doses. The high concentrations of porphyrins in the excreta and the highly significant correlations with liver porphyrins (Figure

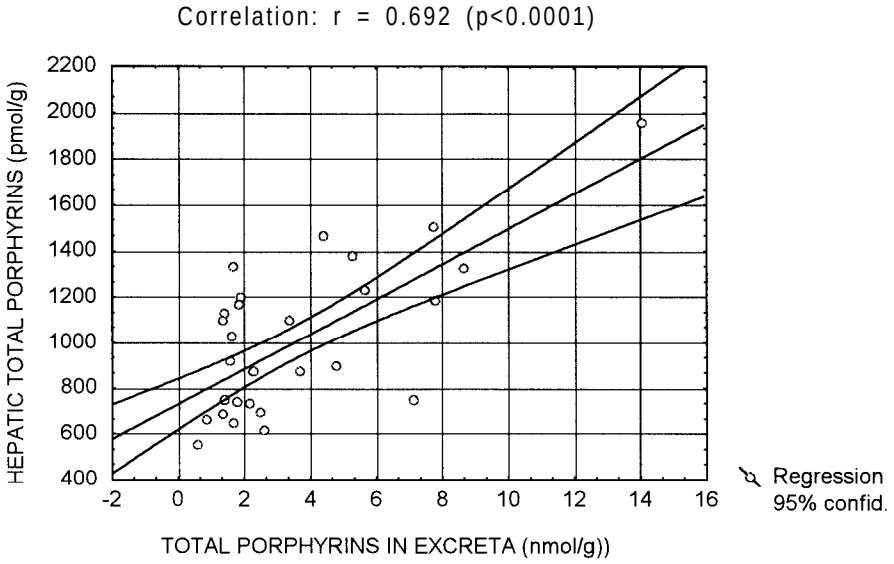

Figure 2. Regression analysis (95% confidence limits) of total porphyrins in the liver and excreta of quails treated with 7 different combination of methylmercury and Arochlor 1260 (control group included) (intercept = 750; slope = 74.5).

2) suggest that excreta can be used as a non destructive sample for the evalutation of risk due to metals and organochlorines. This type of investigation could be performed in birds at risk or close to extinction without sacrificing wildlife. Excreta can be considered an excellent source of information as far as chemical hazards are concerned. In addition to porphyrin analysis, direct determination of contaminats in excreta can also be performed. It was recently shown that metals occur in much higher concentrations in excreta than in conventional storage organs and tissues (Leonzio and Massi, 1989).

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