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Ochratoxin A in Human Blood and Balkan Endemic Nephropathy

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Abstract. The etiology of Balkan endemic nephropathy, a kidney disease encountered among the rural population living in regions along several big rivers on the Balkan Peninsula, remains unknown in spite of many hypotheses put forward and tested. One hypothesis involves mycotoxins as the causal agent. The mycotoxin ochratoxin A has been demonstrated to have a potent nephrotoxic effect in all mammalian species tested so far.

The results of analysis of ochratoxin A in human blood samples by an analytical method based on the measurement of fluorescence spectra, before and after incubation with carboxypeptidase A, is described. For a 2-g-sample the detection limit of the method is 1–2 ng/g serum. High performance liquid chromatography used for the confirmation of ochratoxin A identity by means of several derivatives of the molecule is also described. Out of more than 600 samples collected in an endemic region in Yugoslavia about 7% were positive for ochratoxin A. The highest concentration found was 40 ng ochratoxin A/g serum.

Key words: Ochratoxin A – Kidney disease

Introduction

A fatal human kidney disease encountered in several regions along big rivers in Yugoslavia, Roumania and Bulgaria has been recognized as a special nosological entity in the middle 1950s'. In one among several endemic regions in Yugoslavia the prevalence of the disease varies from 3–8% with a mortality rate of 1–3/1,000 inhabitants/year (Hrabar et al. 1976). The rates have remained practically unchanged in the last two decades (Čeović et al. 1979). The disease represents not only a health, but also a social and economic problem. In spite of

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extensive search the etiology of the disease has remained obscure. Many hypotheses have been advanced, but few have been accepted as credible. Presently the most feasible hypothesis associates fungal metabolites (mycotoxins) including ochratoxin A [(R)-N-(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl) carbonyl phenylalanine, Van der Merwe et al. 1965], with the etiology of the disease (Barnes 1967; Krogh 1974). As demonstrated in several species of experimental animals ochratoxin A exerts a potent nephrotoxic effect (Chu 1974; Krogh 1976; Berndt and Hayes 1979). This secondary metabolite of various common storage fungi is regarded as the main cause of mycotoxic porcine nephropathy, a naturally occurring disease in many countries (Krogh 1978).

Naturally occurring ochratoxin A in human food and animal feed samples was found more frequently in the endemic region than in the countries where the disease is unknown (Krogh et al. 1977; Pavlović et al. 1979) which indicates a higher exposure of both humans and animals in the endemic regions. Since ochratoxin A binds readily to serum albumin (Chu 1971) the presence of the toxin in blood has been suggested as a parameter for the assessment of exposure to this nephrotoxic agent (Hult et al. 1980). An attempt was made to screen human blood samples for the presence of ochratoxin A, using the technique developed and applied to the study of ochratoxin A occurrence in pig blood (Hult et al. 1979). Preliminary results (Pleština et al. 1981) revealed that in 1979 up to 17% of blood samples from a hyperendemic village contained measurable quantities of ochratoxin A.

In this paper the results of ochratoxin A analysis in blood samples collected in a screening campaign which took place in the endemic and non-endemic area in 1980 are described. Special emphasis is given to the confirmation of ochratoxin A identity with high performance liquid chromatography by an examination of ochratoxin A and three ochratoxin A derivatives.

Subjects, Material and Methods

Sampling. The inhabitants of two villages, one hyperendemic (A) and the other in which no clinical cases of nephropathy had been found (B), were taken in the study. Blood samples were collected during the screening campaign in March-April 1980, in which all inhabitants above the age of 3 years were asked to come and to be seen by a medical team. In 90 out of 198-households from village A and in 44 of 80 households from village B, samples were taken from all the inhabitants. In only 24 households from village A and in five from village B no samples were taken. The number of blood samples from each household is given in Table 1. Altogether 420 samples from village A and 219 from village B were analysed. This makes 70% and 81% of the population, respectively.

Blood was withdrawn by a disposable syringe without anticoagulant or preservative. Blood clotted during the shipment to the laboratory (without any cooling) and serum was separated the following day after the samples had been gently centrifuged. The separated sera were frozen at −20 °C until assayed.

The Enzymic Analysis of Ochratoxin A. The method described earlier (Hult et al. 1979) for ochratoxin A in pig blood was followed with slight modifications concerning the volume. A portion of 2 g, or less, of serum was weighed into a centrifuge tube and 10 ml of a solution containing 0.05 M HCl and 0.1 M $MgCl_2$ was added. The mixture was shaken with 5 ml chloroform for 10 min in a tube-turning apparatus. After centrifugation for 10 min at 1,600 *g* 4 ml of the chloroform phase was

Table 1. The number of households as related to the number of samples analysed in a hyperendemic village (A) and a non-endemic village (B)

Number of samples analysed from each household	Village	Number of inhabitants in each household							
		1	2	3	4	5	6	7	8
		Number of households							
0	A	14	8	1	–	1	–	–	–
	B	2	3	–	–	–	–	–	–
1	A	27	18	7	2	–	1	–	–
	B	11	5	–	–	2	–	–	–
2	A		33	9	9	1	1	–	–
	B		14	2	2	–	–	–	–
3	A			11	10	3	5	1	1
	B			5	8	2	–	–	–
4	A				10	5	6	–	–
	B				5	4	–	1	–
5	A					7	–	1	–
	B					6	1	–	–
6	A						2	3	2
	B						2	1	1
7	A							–	–
	B							1	2

transferred to another tube. After washing the chloroform phase once with 1.5 ml water ochratoxin A was extracted with 3 ml 0.04 M tris (hydroxymethyl)aminomethane-sulphuric acid buffer, pH 7.50 at 20 °C. After centrifugation a 2.0 ml portion of the 3 ml buffer layer was transferred to a round borosilicate glass cuvette. The sample was cooled for 10 min on an ice bath. The fluorescence was measured at 380 nm excitation and 450 nm emission. If the signal obtained was lower than the signal from a control cuvette spiked with 2 ng ochratoxin A/g of serum, the sample was stated to contain less than 2 ng ochratoxin A/g of serum and it was not further examined. If the signal was higher than stated above, 100 µl of the enzyme carboxypeptidase A [100 U/ml in 0.04 M tris(hydroxymethyl)aminomethane-sulphuric acid buffer, pH 7.50, 1 M sodium chloride] was added to the sample and the fluorescence excitation spectrum from 320 nm to 400 nm was recorded at 450 nm emission. After incubation for 2 h at 37 °C the spectrum was recorded again after cooling. For all of these measurements a Perkin Elmer Model 204A fluorescence spectrophotometer was used with a polarizing filter installed in the emission pathway between the sample and the emission monochromator. A home-made attachment to the cuvette holder was constructed allowing use of round borosilicate glass cuvettes. If after incubation with carboxypeptidase A the fluorescence at 340 nm increased the loss of fluorescence at 380 nm was used as a measure of ochratoxin A concentration. The recovery of added ochratoxin A (2–75 ng/g serum) from serum samples was 52%. In spite of a rather low recovery the method has a reproducibility and precision better than ± 5%.

Confirmation of Ochratoxin A Identity

The Extraction of Ochratoxin Derivatives. For confirmation of its identity ochratoxin A was extracted from the 1 ml buffer which remained after the sample had been taken for enzymic analysis.

Ochratoxin α (R-5-chloro-3,4-dihydro-8-hydroxy-3-methyl-l-oxo-1H-2-benzopyran-7-carboxylic acid), formed by the enzymic reaction during analysis, was extracted with chloroform after the buffer had been acidified with 25 µl formic acid/ml of buffer. The chloroform was dried over sodium sulphate and evaporated under nitrogen. The residues were dissolved in 125 µl of the eluting solvent to be used for high performance liquid chromatography, or directly for derivatisation.

Fractions containing ochratoxin A and α were collected at the outlet from the high performance liquid chromatography column. To each millilitre of the fractions 25 µl formic acid and 1 ml chloroform were added and the ochratoxins were extracted. In the case of ochratoxin A 0.5 ml water was added per millilitre of sample to obtain a two-phase system. The water phases formed during extraction were discarded. The chloroform phases were dried over sodium sulphate and evaporated under nitrogen.

Derivatisation. To the dry samples of ochratoxin A and α 1 ml of methanol with 10% boron trifluoride was added and the samples were kept at 65 °C for 30 min (ochratoxin A) or 60 min (ochratoxin α). After cooling 1 ml of water was added to each sample and the esters were extracted with 1.5 ml of chloroform. The chloroform was evaporated under nitrogen. The residue was dissolved in 125 µl of the solvent used for chromatography.

High Performance Liquid Chromatography Apparatus. An Altex pump, model 110 and an Altex injector equipped with a 100 µl loop were used. The column was LiChrosorb RP 18, 10 µm (250 × 3.3 mm) fitted with a home-made water jacket attached to a Circulator C-400 thermostator from Techne, Cambridge. The detector was a Schoeffel Model FS 970 L.C. fluorometer. Excitation wavelength 340 nm, excitation filter Corning 7–54 and a cut-off emission filter 470 nm were used. For the detection of ochratoxin A methyl ester in the alkaline eluent (III) the excitation wavelength was changed to 380 nm.

Eluents. The following three eluents were used:

I Methanol-water-formic acid (50 : 50 : 2) for ochratoxin α and its methyl ester;
II Methanol-water-acetic acid (70 : 30 : 2) for ochratoxin A and its methyl ester;
III Methanol-0.1 M sodium acetate (60 : 40) for the two methyl esters.

Standards. A stock solution of authentic ochratoxin A (a kind gift form Dr P.S. Steyn, Pretoria, South Africa) was prepared in 0.04 M tris(hydroxymethyl)aminomethane-sulphuric acid buffer, pH 7.50 (20 °C). The concentration was calculated from the absorbance at 380 nm using the molar extinction coefficient 5,680 $M^{-1} \cdot cm^{-1}$ (Hult et al. 1979).

Ochratoxin α was prepared from ochratoxin A by enzymic hydrolysis. Ester standards were prepared from ochratoxin A and α standards, according to the methods described above.

Results

Enzymic Analysis

The detection limit of the enzymic method for ochratoxin A analysis is 1–2 ng ochratoxin A/g of serum. To reach this detection limit 2 g of serum is needed. Unfortunately, many samples were less than 2 g, so that for these samples a higher detection limit was accepted (up to 8 ng/g serum). The results of enzymic analysis are shown in Tables 2 and 3.

High Performance Liquid Chromatography

Table 4 shows the results obtained when the ochratoxin A containing samples were analysed with the method of high performance liquid chromatography in

Table 2. The number of samples not containing ochratoxin A as found by the enzymic method with respective detection limits

Detection limit ng/g serum[a]	Village A	Village B
2	157	93
3	160	74
4	62	6
5	14	28
8	2	1
Total	395	202

[a] Low level of ochratoxin A were not possible to detect in all samples, as some samples contained less than 2 g of sera, which is needed to reach the lowest detection limit

Table 3. The number of samples containing ochratoxin A as found by the enzymic method

Ochratoxin A ng/g serum	Village A		Village B	
	N	% positive	N	% positive
1– 2	11	2.62	4	1.83
3– 5	11	2.62	7	3.20
6–10	2	0.48	6	2.74
> 11	1	0.24	0	0
Total	25	5.95	17	7.76

addition to the enzymic method. The four high performance liquid chromatograms for a sample taken from village B, containing 8 ng ochratoxin A/g serum according to the enzymic method, are shown in Fig. 1.

The free carboxylic acid groups of ochratoxin A and α, have dissociation constants so different that the two substances are not conveniently chromatographed on a reversed phase with the same solvent system. The stronger acid, ochratoxin α, is not retained when a suitable solvent pH for ochratoxin A is used. On the other hand ochratoxin A has too long a retention time in a system with the low pH needed for ochratoxin α chromatography. One problem arose when chromatographing the methyl ester derivatives prepared from free acid derivatives collected from the effluent of the chomatography column. Only after this collection and derivatisation did a ghost peak appear overlapping the ester peaks. This happened only in the acid systems (I and II), but caused no problem in the alkaline system (III). If the esters were prepared without any previous chromatography the ghost peak did not appear.

The formation of a carboxylic ester during the derivatisation of ochratoxin α with methanol-boron trifluoride is consistent with the change in solubility properties and chromatographic behaviour of the obtained derivative. The fluorescence characteristics confirm the presence of a free hydroxyl group by the shift of the excitation maximum from 340 to 385 nm due to the change in pH from 2 to above 10.

Table 4. Analysis of ochratoxin A in human sera by the enzymic and HPLC method. Concentrations of ochratoxin A derivatives determined in the same samples by the HPLC method are expressed in nanograms of ochratoxin A per gram of serum. For ochratoxin A determined the HPLC method and for its methyl derivative eluent methanol-water-acetic acid (70:30:2) was used. For ochratoxin α and its methyl derivative eluent methanol-water-formic acid (50:50:2) was used

Village	Ochratoxin A		Ochratoxin derivatives		
	Enzymically	HPLC	HPLC OA-methyl	Oα-methyl	Oα
	ng ochratoxin A/g serum				
A	3	2	–	4	–
	3	1	–	–	2
	3	3	–	–	–
	5	4	6[a]	5	4
	2	–	–	–	3
	40	57	42	43	44
	6	–	–	7[a]	5
	10	12	–	8[a]	7
	3	–	–	5	–
	2	–	–	6	–
	3	2	5[a]	–	2
	6	–	–	–	7
B	5	2	–	–	5
	5	–	–	7[a]	4
	8	9	13[a]	9[a]	6
	3	1	–	–	5
	8	3	–	11[a]	6
	6	4	–	6[a]	3
	5	–	–	–	5
	4	–	–	–	5
	3	2	–	–	2
	7	–	–	–	7
	6	3	–	6[a]	4
	5	4	–	5[a]	3

[a] Alkaline methanol-0.1 M sodium acetate (60:40) eluent used

Discussion

The method used for ochratoxin A analysis in a large number of samples is based on the hydrolysis of the amide bond in the molecule catalysed by the enzyme carboxypeptidase A (Hult et al. 1979). The method does not depend on a specific substrate-enzyme relationship as ochratoxin A is not the only substrate for the enzyme. Instead, the specificity of the method is based on the chemical and physical properties of the ochratoxin molecule. The most important properties used are the fluorescence characteristics in alkaline buffers of the two ochratoxin derivatives A and α. The change of the fluorescence excitation spectrum during hydrolysis is caused by the properties of the ochratoxins revealed after the hydrolysis of the amide bond by the enzyme carboxypep-

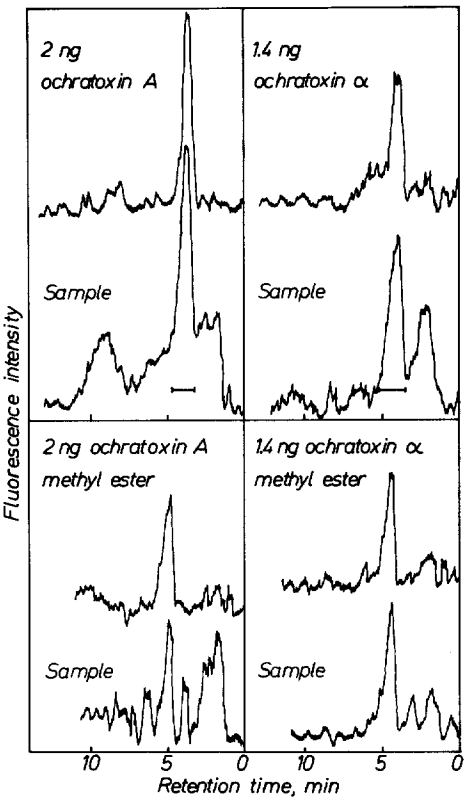

Fig. 1. High performance liquid chromatograms of a sample taken from village B estimated by the enzymic method to contain 8 ng ochratoxin A/g of serum. The derivatives ochratoxin A and α were recovered from the reaction mixture of the enzymic analysis in order to be used for chromatography. The derivatives ochratoxin A methyl ester and ochratoxin α methyl ester were prepared from ochratoxin A and α respectively collected from the chromatography column effluent. The times of collection are indicated by bars. The flow rate was 1 ml/min

tidase A. Other properties of the ochratoxin A molecule that are important for the specificity of the method are those used for the extraction steps.

Further confirmations of ochratoxin A identity have been done by high performance liquid chromatography of four different ochratoxin derivatives (Table 4). Ochratoxin A was recovered from surplus buffer extract, which was not used for the enzymic analysis. For some samples it was possible to recover ochratoxin A from the chromatographic step and prepare the ester derivative before a new chromatographic step. The use of the ester derivative for confirmation of ochratoxin A identify in chromatographic systems has become a standard procedure (Nesheim et al. 1973; Josefsson and Möller 1979; Hunt et al. 1980). To take further advantage of the enzymic analysis ochratoxin α was recovered from the buffer after the enzymic hydrolysis and was used for chromatography. The methyl ester derivative of ochratoxin α was prepared from either the whole sample, or a part of the sample, or from ochratoxin α collected after chromatography.

The confirmatory methods described above have been applied to a total number of 24 human serum samples, resulting in the confirmation of ochratoxin A identity and quantity in all samples analysed (Table 4); ochra-

toxin A was used for 15 samples, ochratoxin A methyl ester for 4, ochratoxin α for 20 and ochratoxin α methyl ester for 13 samples. Two different solvent systems, one acidic and one alkaline, were used for the chromatographic analysis of the two ester derivatives. On the whole ochratoxin A was identified in six different modes, besides the enzymic method, all including quantitation.

The results of ochratoxin A occurrence in human serum presented here (Table 3) refer to the samples collected during March-April 1980, and cover 70% and 81% of the total population in the hyperendemic and non-endemic village. The rates of ochratoxin A occurrence among individuals in the two villages are similar: 6.0% and 7.8%.

In 1980 the percentage of serum samples containing ochratoxin A in the hyperendemic village (A) was considerably lower than in 1979 i.e., 6.0% against 16.6% (Pleština et al. 1981). In the non-endemic village (B) the frequency remained practically unchanged, 7.8% in 1980 and 7.6% in 1979. It should be pointed out that the analyses carried out in 1980 were performed under the conditions which caused a decrease in the sensitivity of the method. This was mainly due to small quantities of sera available for analyses but partly also to the procedure used in which only the samples with a certain minimal fluorescence were incubated with the enzyme and measured twice, as described above. The latter saved a lot of time, but decreased the probability to find samples with ochratoxin A in the range 1–2 ng/ml. Thus while in 1979 even 63% of positive samples were within the range 1–2 ng/ml of serum, in 1980 only 36% of positive samples were within this range.

Although uniform distribution of ochratoxin A containing samples from both villages in 1980 would not appear to support the involvement of this nephrotoxic agent in Balkan endemic nephropathy its role cannot be ruled out. This is largely due to known yearly fluctuations in the presence of ochratoxin A in human environment. Moreover, a mild kidney damage regardless of the cause could not be excluded with the screening methods used at present. In certain years an unexplained high prevalence of albuminuria was recorded in this village (own unpublished results) and therefore, although no clinical cases of nephropathy have been recorded, the village cannot be taken as a control for this type of study. However, the results confirm the practicability of the enzymic method for the screening of a large number of people. By such regular monitoring for the presence of ochratoxin A in selected population groups over a longer period of time concurrently with an epidemiological survey it will be possible to assess its role in the aetiology of this fatal disease.

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