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OCHRATOXIN A IN MATERNAL AND FOETAL BLOOD AND IN MATERNAL MILK

OCHRATOKSYNA A W SUROWICY KRWI MATKI I PŁODU ORAZ MLEKU MATKI

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Ochratoxin A (OA) levels were measured in blood serum of mothers foetuses and in mothers' milk. The mean concentration of OA in maternal serum was 1.14 ng/ml and in umbilical cord blood serum it was 1.96 ng/ml. The mean ratio of OA concentrations in maternal and foetal blood serum was 1.96. In maternal milk OA was found in 5 out of 13 studied samples. The mean intake of OA with mother's milk was not exceeding the tolerable daily intake for adults, nevertheless it was near 60% of TDI. The OA concentration ratio in maternal blood serum versus that in milk was 0.0058 on average. These results confirm the correlation between OA concentration maternal and foetal blood serum, and between OA concentration in maternal serum and milk.

Key words: ochratoxin A, determination, HPLC, blood serum, maternal milk
Słowa kluczowe: ochratoksyna A, oznaczanie, HPLC, surowica krwi, mleko matki

INTRODUCTION

Ochratoxin (OA) is a compound belonging to mycotoxins – large group of secondary metabolites of moulds. They can cause the acute intoxication and frequently exhibit mutagenic, carcinogenic, teratogenic and estrogenic properties. In temperate and cold climate OA is produced by *Penicillium verrucosum*, and warm or tropical climate also by certain *Aspergillus* species [18].

Ochratoxin A is a substance with broad spectrum of harmful effects including nephrotoxicity. OA is believed to be a factor causing urinary tract neoplasms in humans and so called Balkan Endemic Nephropathy (BEN) [21]. Reports from Near East and France suggest that OA can be the cause of chronic interstitial nephritis [5, 23, 29]. OA exerts also teratogenic effects [1], as well as immunotoxic [28], and possibly neurotoxic action [17].

In 1993 the International Agency for Research onCancer (IARC) classified OA as a compound potentially carcinogenic for human (group 2B) [12]. In 1991 and 1995 the Joint Expert Committee for Food Additives FAO/WHO (JECFA) evaluated the toxicity action of OA for humans and established Provisional Tolerable Weekly Intake (PTWI) as 100 ng/kg b.w./week [13].

The Canadian authorities suggest accepting of the permissible daily intake as 1.2-5.7 ng, OA/kg b.w. A similar value has been postulated by the Nordic Committee of Food Toxicology which proposed the highest Tolerable Daily Intake (TDI) of 5 ng OA/b.w. The EU Scientific Committee for Food (SCF) established the TDI value below 5 ng OA/kg b.w. [7, 15].

OA was detected in human milk also in some countries [2, 9, 11, 14, 16, 19, 20, 27, 31]. This shows that toxin is ingested with food can pass into human milk. OA transfer across the placental barrier was demonstrated also by *Zimmerli* and *Dick* [31]. Thus the harmful effects of OA may endanger not only the mother but also the foetuses and the breast fed infants [26, 30].

MATERIALS AND METHODS

The material was obtained from women according to the principles of the Good Clinical Practice. The consent for carrying out these studies was obtained from the Committee for Supervision of Human Experiments at the Mother and Child Institute.

Samples of milk, maternal blood and umbilical cord blood were obtained between October 1998 and April 1999. Samples blood, umbilical cord blood and human milk were obtained from 30 women in labour – healthy patients of the Mother and Child Institute. Maternal venous blood was taken in 5 ml aliquots into dry test tubes immediately before labour. No blood samples were taken from patients receiving large volumes of infusion fluids. For the assessment of foetal exposure 5 ml samples of blood were taken from the umbilical cord into dry tubes immediately after cord cutting. The samples were centrifuged for 10 minutes at 2500 g and the obtained serum was frozen at -18°C until performing of the determinations.

Milk samples were taken from the same patients from whom blood samples had been taken previously. Milk was obtained on 3-4 days after labour; the samples were frozen and kept at -18°C until determinations.

Immunoaffinity columns (IAC) OchraTest, Vicam, USA, and OchraPrep, Rhone-Poulenc Diagnostic, UK were used. The ochratoxin A standard was obtained from Sigma, USA. The solvents used for chromatography was of purity for HPLC, the remaining solvents were pure for analysis. The water used was purified by reverse osmosis and demineralisation.

Ochratoxin A determination

For the determination of ochratoxin A in human milk and blood serum *Zimmerli* and *Dick* method was used in own modification [31].

10 ml of a solution composed of 80% H_3PO_4 33.7 ml, NaCl 118 g in 1 l of water, pH 1.6 was added to 5 ml of milk or serum. The sample with the solution was mixed on vortex for 1 minute and centrifuged for 10 minutes at 2500 g. Then 5 ml of chloroform was added and the sample was mixed on vortex for 1 minute and centrifuged for 10 minutes at 2500 g. The extraction was repeated four times. The cumulated chloroform phases were dryed in rotary evaporator at 40°C and the residue was transferred quantitatively with 6 ml $CHCl_3$ into a tube with stopper. Ochratoxin was re-extracted three times with 5 ml portions of 1% $NaHCO_3$ solution and was load onto IAC column.

The pulled extracts were passed through IAC column at flow rate of 1-2 drops/s. The column was washed with 10 ml of a PBS-0.01% Tween 20 solution and then with 10 ml of water at flow 1-2

drops/s and then several ml of air was passed though the column. Ochratoxin A was eluted with 3 ml of methanol of HPLC purity at flow rate 0.5 drop/s. Methanol eluate was evaporated in water bath at 40°C under nitrogen stream, the dry residues was dissolved in 50 µl of the mobile phase and loaded into the chromatograph.

HPLC condition

The HPLC set Waters, USA, with Waters 474 scanning fluorescence detector was used. The column was RP C18 Nova Pak C 18, 4.6 x 250 mm, Waters. The column was thermostated in 50°C. Behind the column a connecting three-piece was installed for ammonia solution passing to increase fluorescence (Waters M501 HPLC pump with high pressure noise filter, Waters).

Conditions in detail: mobile phase: methanol – 9% acetic acid (18-7), mobile phase flow rate 1 ml/min, column temperature 50°C; detector Ex wavelength λ = 390 nm; Em wavelength λ = 440 nm, 20% ammonia solution flow rate 0.2 ml/min, injection volume 20 µl.

The obtained positive results were confirmed by the formation ochratoxin A methyl ester. The same conditions of determination were used as for OA [31].

RESULTS AND DISCUSSION

Analytical parameters of the method

The method used was tested for its analytical characteristics determining the recovery, relative standard deviation (RSD), and the limits of detection and determination. Two types columns with monoclonal antibodies OchraTest, Vicam, and OchraPrep, Rhone-Poulenc Diagnostic were compared. The analytical parameters for OA determination in milk are presented in Table I.

Table I. Analytical parameters of methods

Matrix	Fortification level [ng/ ml]	Number of samples	Recovery [%]	RSD [%]	Limit o detection [ng/ ml]	Limit of determination [ng/ ml]
Milk	0.02[1]	8	81	11	0.005	0.015
	0.02[2]	4	89	16	0.005	0.015
Blood serum	0.5	6	79	9.1	0.02	0.06

[1] OchraTest, Vicam

[2] OchraPrep Rhone-Poulenc Diagnostic

The results of the determinations were compared using *Student's* t test at P>0.05 confidence level. No statistically significant differences were found between the results of the determinate using Vicam or Rhone-Poulenc Diagnostic columns.

The modification included simplified extract purification through elimination of the liquid-liquid extraction phase. The analytical parameters obtained (e.g. for human milk, 5 ml sample, limit of detection was 0.005 ng/ml, RSD 11-16%, mean recovery 85%) were similar to those described in the original method (0.005-0.01 ng/ml, RSD recovery 85% respectively).

An advantage of the modification was shortening of analysis time, reduced work expenditure and amount of organic chlorine solvent harmful for the environment, used for extraction.

Determination of mycotoxins in food and biological materials is regarded as difficult, and the methods used are often costly laborious. The problems are increased when additional difficulty appears, e.g. limited availability of biological material for tests which precludes the possibility of performing of a great number of determinations, as is often occurring in tests of material derived from human beings.

Ochratoxin A in maternal and umbilical cord serum and in mater milk

In the studies blood and umbilical cord blood samples were obtained parallelly from the same patients. The mean OA concentration in blood serum from 30 patients was 1.14 ng/ml, median 1.00 ng/ml. The mean concentration in umbilical cord blood serum (28 samples) was 1.96 ng/ml, median 1.83 ng/ml OA concentration ratio in maternal blood serum to that in umbilical cord blood serum was 1.96 on average, with 0.6-4.0 range. The obtained results are presented after correction for recovery. The obtained numerical value was accepted for the values of concentrations between limit of detection and limit of determination for further calculations. A statistically significant difference was found between the mean OA concentration in maternal blood serum and umbilical cord serum (*Student's* t test > 0.05). These results are presented in Table II.

Milk samples were obtained from 13 patients. Ochratoxin A was found in five. The mean ratio of OA concentration in maternal blood serum to that in milk was 0.0058.

In the literature as yet only one study has been found on OA concentrations in maternal and foetal blood serum. The value of this ratio is the same as in the study of *Zimmerli* and *Dick* [31] (2.0±0.2) but the scatter of the values was much broader (1.96±0,96). The ratio calculated for OA was similar to that calculated for phenylalanine levels in maternal blood and umbilical cord blood which ranged from 1.2 to 1.9 [25]. The higher OA content in foetal blood could be explained as due to its active transport across – the placenta, perhaps this was OA consequence of similar chemical structures of OA and phenylalanine [4].

The foetus receives all nutrients and other alimentary component through the placenta, which is also a barrier protecting against the penetration of harmful substances. Chemical compounds of low molar mass, as well as those with lipophilic character, can pass however, through the placental barrier. Certain components, e.g calcium, can be actively transported across the placenta. The infantile organism is particularly sensitive to xenobiotics, especially in the first 5 months of life, mainly due to insufficiently adequate functions of the kidneys and hepatic metabolism system. In infants glomerular filtration is about 1/3 lower in relation to adults. In the liver alcohol dehydrogenase is not yet fully developed, the levels of dehydroxylation and oxidation are also lower. That means that TDI or values obtained in adults in toxicological studies could not be applicable to infants [22].

In the literature only *Breitholtz-Emanuelsson* et al. [2] report the value of milk/blood ochratoxin A ratio as being below 0.1, that is below 0.05 if the ratio of concentrations in whole blood and serum being 2, is considered. This value is similar to that obtained in the presently reported study.

In animal experiments on rats and rabbits OA was found to pass to milk [3, 8]. Giving OA 10-250 ng/kg b.w. to rats the milk/plasma ratio was 0.2-0.5, and it was higher than in rabbits. The mechanism of OA transfer to milk is not known. Passive diffusion of free, non-ionized OA is possible. In the case of that mechanism the transport would depend on pH of the plasma and milk. The differences in milk/blood ratios could be due to interspecies varia-

Table II. Ochratoxin A contents in maternal and foetal serum blood and in maternal milk

No.	Maternal blood serum [ng/ml]	Foetal blood serum [ng/ml]	Maternal blood/ Foetal blood	Milk [ng/ml]	Maternal blood/ Milk
1	3.41	5.42	1.6	0.017	0.005
2	0.81	2.20	2.7	—	—
3	1.36	2.23	1.6	—	—
4	2.11	1.26	0.6	—	—
5	1.07	0.94	0.9	—	—
6	1.29	3.57	2.8	—	—
7	1.15	0.00	—	—	—
8	2.39	1.48	0.6	0.0053	0.002
9	2.40	3.87	1.6	—	—
10	1.00	1.70	1.7	—	—
11	1.11	3.77	3.4	—	—
12	0.14	0.57	4.0	—	—
13	2.05	4.02	2.0	0.014	0.007
14	1.53	4.04	2.6	n.d	—
15	0.50	1.85	3.7	n.d.	—
16	0.51	0.68	1.3	—	—
17	2.15	2.28	1.1	0.006	0.003
18	1.00	0.76	0.8	n.d.	—
19	0.33	1.06	3.2	—	—
20	0.85	1.69	2.0	—	—
21	1.22	2.13	1.8	nd.	—
22	0.57	1.99	3.5	n.d.	—
23	0.63	0.82	1.3	n.d.	—
24	0.43	0.56	1.3	n.d.	—
25	0.37	0.80	2.1	—	—
26	0.64	0.97	1.5	—	—
27	0.47	0.76	1.6	n.d.	—
28	0.82	0.00	—	0.009	0.012[1]
29	0.91	1.46	1.6	—	—
30	1.11	2.12	1.9	—	—
Mean	1.14	1.83	1.96	0.0056	0.0058
SD	0.76	1.35	0.94	0.004	
Median	1.00	1.59			

bility of proteins in plasma and milk. Literature data on the correlation between blood OA levels and its penetration into milk are scant and the problem of OA presence in milk in relation to lactation time should also be clarified. No data are available on OA elimination with milk after repeated administration of small doses.

Ochratoxin A presence in blood is a generally accepted evidence of the exposure to it. The presently reported values are similar to those reported from other European countries [6].

The possibility of daily OA intake estimation based on its blood level, biological availability and clearance is very important. Two possibilities are available for estimation based on OA clear value. *Hagelberg* et al. found it to be 0.033 ml/min. In humans this clearance was determined experimentally and its value was 0.048 ml/min. It is worth stressing, however, that this result was obtained in the experiment on one volunteer. OA bioavailability in humans is not known, and in the calculations the value determined in rats, that is 50% has been accepted [10].

The daily intake of OA estimated on the basis of blood OA level is 1.53 ng/kg b.w. (according to the coefficient calculated in [10]) or 2.2 ng/kg b.w. (according to the coefficient of *Schlatter* [24]). It should be stressed that the values of daily intake calculated on the bas of serum OA concentration are not exceeding TDI value. The maximal OA value (3.42 ng/ml) corresponded to OA concentration in diet, which was dependent on the method of determinate being 4.5 ng/kg b.w. daily or 6.7 ng/kg b.w. daily. These values are similar to those established by the Nordic Committee.

In view of the ubiquity of ochratoxin A, its very broad spectrum of harmful activities and long half-time in food products, as well as the sensitivity of the organism, the problem of exposure of the foetus as well as infant to this toxin should be regard as serious. Mothers milk should not contain any noxious substances in view of the impossibility of establishing of a control over human milk, the only known in practice, method for OA intake reduction is limitation of its presence in food through adherence to good manufacturing practice (GMP) and the application of the hazard analysis critical control points (HACCP) on all stages of food product. Particularly important would be systematic monitoring of raw materials and food products that are the main source of ochratoxin in diet, especially cereals and their products.

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OCHRATOKSYNA A W SUROWICY KRWI MATKI I PŁODU ORAZ W MLEKU MATKI

Streszczenie

Zbadano zawartość ochratoksyny A (OA) w surowicy krwi matki, płodu i w mleku. Średnie stężenie w surowicy krwi matki wynosiło 1,14 ng/ml, a w krwi pępowinowej 1,96 ng/ml. Stosunek stężeń OA w surowicy krwi matki i krwi pępowinowej wynosi średnio 1,96. W mleku matki wykryto OA w 5 na 13 zbadanych próbek. Średnie wartości dziennego pobrania OA z mlekiem matki nie przekraczają tolerowanego dziennego pobrania ustalonego dla osób dorosłych, niemniej osiągają one wartość około 60% TDI. Stwierdzono, że stosunek stężeń OA w surowicy krwi matki do mleka wynosi średnio 0,0058. Uzyskane wyniki potwierdzają istnienie korelacji między stężeniem ochratoksyny A w surowicy krwi matki i surowicy krwi płodu, a także między stężeniem ochratoksyny A w surowicy krwi i mlekiem matki.

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