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Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data

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Abstract

An improved specific analytical method for ochratoxin A (OA) is presented, combining HPLC separation with enhanced fluorescence detection by post-column addition of ammonia. Commercial immunoaffinity columns (Biocode) were for the first time applied to the cleanup of extracts of body fluids; they could be used up to 20 times for blood serum. The extraction efficiency of OA from human serum and milk as well as its derivatization to esters were studied and improved. The quantitation limit for OA was improved and estimated at 5–10 pg/g for human milk and serum. The mean recovery of OA from serum and milk was estimated at 85%. The overall coefficient of variation for OA determinations in serum, milk and selected foodstuffs was estimated at 10% (concentration range 0.01–5 ng/g). The method was applied to sera of 368 blood donors, 10 pairs of maternal and fetal sera, as well as to 40 human milk samples and selected foodstuffs; the results are discussed.

1. Introduction

The secondary fungal metabolite ochratoxin A [OA; (*R*) - N - [5 - chloro - 3,4 - dihydro - 8 - hydroxy - 3 - methyl - 1 - oxo - 1H - 2 - benzopyran - 7 - yl)carbonyl] - L - phenylalanine; $C_{20}H_{18}O_6NCl$ (403.8 g/mol); CAS Reg. No. 303-47-9] is produced by several molds belonging to the genera *Aspergillus* (e.g. *A. ochraceus*) and *Penicillium* (e.g. *P. verrucosum*) [1–3].

OA has a well documented nephrotoxic effect in several monogastric domestic and experimental animals (e.g. porcine nephropathy). It has also demonstrated to have weak genotoxic, carcinogenic, teratogenic and immunosuppressive properties in experimental animals. Risk assessments of OA by various organizations and authors based on various criteria resulted in a maximum tolerable daily intake for humans in the range 0.2–16 ng/kg body weight (b.w.) [1–3]. In view of its potential carcinogenicity in humans a daily intake of OA in the order of 5

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ng/kg b.w. may be a reasonable estimate for a "virtually safe dose" (VSD).

In order to evaluate the possible human health risks of OA, an estimate of its daily intake by the population and/or its concentration in the blood is needed. This procedure has several advantages: firstly, only one type of sample has to be analysed; secondly, as a consequence of the estimated relatively long half- life of OA in human blood of 20–50 days (strong binding to serum proteins; probable enterohepatic circulation), the OA concentration in the blood should be higher than in the daily diet.

Many different methods for the analysis of OA in feed, foods and body-fluids have been described in the past. In some recent papers the analytical methodology of OA has been reviewed [4,5]. The methods are usually based on high-performance liquid chromatography (HPLC) on a reversed-phase (RP) column with an acidic eluent [6–11] or at slight alkaline pH using an ion-pair technique [12,13] and fluorescence detection. Mass spectrometry has also been coupled to HPLC separation [14]. Published quantitation limits of OA in body-fluids average at ca. 100 pg/ml, limits of 5–50 pg/ml have been recently reported for the most sensitive chemical methods [11,15].

Several authors recently described the very promising use of immunoaffinity columns (IAC) for the cleanup of OA-containing extracts [3,10,16]. This adaptation of immunological methods has been very successful for aflatoxins [17–19], but their commercial availability for other mycotoxins has been limited up to now.

The aim of this work was to develop a HPLC procedure for the sensitive and reliable determination of OA in human serum samples, and its use to estimate the exposure of the Swiss population to OA. A limit of quantitation of OA of ca. 5 pg/ml with a maximum sample size of about 2 ml serum, including a quantitative confirmation step, was therefore envisaged. Our approach was to combine and optimize existing techniques in OA analytical methodology. After serious problems emerged with blank values, we switched from silica-gel adsorbents to new experimental IACs (Biocode) with monoclonal antibodies against OA for sample cleanup. In the mean time these became commercially available and were recently used in an automated liquid chromatographic method for OA in cereals and animal products [10].

2. Experimental

2.1. Reagents

OA in crystalline form was purchased from Aldrich (Milwaukee, WI, USA) or Sigma (St. Louis, MO, USA), and ochratoxin B (OB) from Food Science and Technology CSIR (Pretoria, South Africa). For HPLC a working standard of 7 ng/ml dissolved in the HPLC solvent (methanol–water–acetic acid) was used. The very diluted standard solutions of OA were always carefully handled and stored most of the time in the refrigerator protected from light. No indication of OA decomposition over a period of several months was observed. However, in alkaline solution its stability is limited. Methyl ester standards of OA and ochratoxin α (see derivatization) were prepared by esterification of the corresponding OA standards.

The stock solution was prepared by dissolving 0.7 mg OA in 100 ml of methanol. It was stored at ≤ -20°C. The purity of the standard was checked spectrophotometrically at a concentration of 7 μg/ml in benzene–acetic acid (99:1, v/v), assuming an $\epsilon = 5550$ l/mol/cm at $\lambda = 333$ nm and a molar mass of 403 [20]. The purity of the OA standard (Aldrich) was 82.7%. This quantitative calibration of the standard is critical because different suppliers of OA do not always state the exact purity and it seems that most of them offer OA containing about 15% (w/w) benzene.

The IACs (EASY-EXTRACT) were supplied by Biocode (Heslington, York, UK) and the chemicals to prepare the phosphate buffered saline (PBS) solution (NaCl 120 mmol/l, KCl 2.7 mmol/l, phosphate buffer 10 mmol/l, pH 7.4) by Sigma (Diagnostics No 1000-3, St. Louis, MO, USA). To the PBS solution 0.5 g/l of sodium azide was added as a preservative. All other reagents except methanol (Mallinckrodt

Nanograde, Oryx Pharmazeutika AG, Zurich, Switzerland or Merck Lichrosolv, gradient grade, Merck, Darmstadt, Germany) were p.a. grade and used without further purification. Deionized water was used throughout this study.

2.2. Apparatus

The chromatographic system consisted of a Model 420 pump (Kontron Instruments, Zurich, Switzerland), a Model U6K injection valve (Waters, Milford, MA, USA), a Model 650-10S fluorescence spectrometer (Perkin-Elmer, Norwalk, CT, USA) commercially equipped with a 20-μl flow-cell and a 150-W short arc xenon lamp (excitation 390 nm, emission 440 nm, slit width 20 nm). A Model 100A pump (Altex, Berkeley, CA, USA) was used to add a stream (0.1 ml/min) of 25% ammonium hydroxide solution (Fluka No. 9860, puriss., Buchs, Switzerland) to the column eluate before it passed into the detector. The connections were of stainless steel tubing (0.18 mm I.D.) with a low dead-volume T-piece (Valco); the length of the "reaction coil" was about 20 cm. To measure peak areas and/or heights, a Model D-2000 chromato-integrator (Merck-Hitachi, Tokyo, Japan) was used. A prepacked stainless steel column (250 × 4.6 mm I.D.) with 5 μm Spherisorb ODS-1 (Phase Sep, Phase Separation, Norwalk, CT, USA) and a guard column (30 × 4.6 mm I.D.) packed with 5 μm Spherisorb ODS-2 (Phase Sep) were used.

2.3. Chromatographic conditions

The mobile phase (0.45-μm filtered and degassed) consisted of methanol and 9% (v/v) glacial acetic acid in Nanopure water (pH 2.3) (typically 18:7, v/v), at a flow-rate of 1 ml/min. The column temperature was kept at 50°C (including the inlet tube from the injector and the guard column); typical injection volumes were 20 μl. A maximum volume of 70 μl OA dissolved in the mobile phase could be injected without causing a decrease in the number of theoretical plates. The apparent pH of the eluent (glass electrode) was 3.5 before the T-piece and 9 after the detector. The retention times of OA and its methyl ester under the conditions described were approximately 5 and 6.5 min, respectively. For quantitation peak heights were measured and compared to those of appropriate standard solutions in the range of 0.01–15 ng. They were linearly related to the injected (20 μl) amount of OA.

By washing the column daily with mobile phase and methanol, and replacing the guard column as soon as the back-pressure significantly increased, we were able to use the same column for almost three years (several thousand injections), without any marked reduction of plate height or peak distortion.

2.4. Samples

During the development of the method blood, serum and plasma samples from pigs (slaughterhouse) as well as pooled samples of human serum (hospital) and cow milk were used. The human serum samples used in the exposure study were kindly provided by various regional sections of the Blood Donors Service of the Swiss Red Cross during the period of November 1992 to June 1993. The exact dates of sampling were not always available to us. The donors, 205 men and 163 women, were between 20 and 40 years old and were residents from different parts of the country. The human milk samples were a generous gift of the Kantonales Frauenspital, Neonatology Department, Berne (Prof. A. Moessinger); they were collected between August 1992 and February 1993 (north of the Alps). Dr. A. Malek (Placental Perfusion Laboratory) at the same institute kindly provided the maternal and umbilical cord serum samples to us. All samples arrived at our laboratory frozen and were stored at ≤ -20°C until analysed. All extracts of serum and milk samples were analysed on the same day as they were prepared.

2.5. Extraction

Blood and serum

A solution containing 33.7 ml of 85% orthophosphoric acid (p.a. Merck No. 573, density 1.71 g/ml) and 118 g (2 mol) sodium chloride (Fluka No. 71380, puriss p.a.) per liter (pH without sodium chloride 1.6) was prepared. A

10-ml volume of this solution was added to a sample of 0.5–2 g human serum (or blood) in a 30-ml glass centrifuge tube with a PTFE-lined screw-cap (10 cm × 2.5 cm O.D) and intensively mixed for 1 min (Vortex). After addition of 5 ml of chloroform and intensively mixing for 2–3 min, the mixture was centrifuged at 2500 *g* for 15 min. A compact thin layer formed between the two phases. The clear organic phase at the bottom of the tube was carefully withdrawn by pasteur pipette and transferred to a pear-shaped flask (150 ml). The extraction was repeated with another 5 ml of chloroform and the combined extracts were evaporated to dryness at 30–40°C (Rotavapor).

Milk

To 5 g of human milk, 10 ml of the H_3PO_4–NaCl solution (see blood and serum) was added, intensively mixed for 1 min, and extracted four times with 5-ml portions of chloroform (see blood and serum). The combined chloroform extracts were collected in a pear-shaped flask (150 ml) and evaporated to near dryness (Rotavapor, 30–40°C).

The residue was quantitatively transferred with small portions of chloroform (total 5 ml) to a 10-ml stoppered graduated cylinder and extracted twice with 5-ml aliquots of sodium bicarbonate solution (10 g/l p.a. Merck No. 6329) to separate OA from the milk fat. In order to remove interfering substances, the bicarbonate solution (200 ml) was extracted three times with 10 ml of chloroform each prior to use. The combined aqueous phases were pipetted into a separating funnel (60 ml) containing 0.5 ml of concentrated formic acid (98–100%, Merck No. 264) and 1 ml of chloroform. The aqueous phase was then back-extracted four times with 1 ml of chloroform. The chloroform extracts were collected in a pear-shaped flask (150 ml) and evaporated to dryness (Rotavapor, 30–40°C).

2.6. Immunoaffinity cleanup

This step was carried out according to the protocol of Biocode: the residue of the chloroform extract was dissolved in 5 ml of PBS solution containing 15% (v/v) methanol by thoroughly rinsing the walls of the pear-shaped flask and quantitative transfer to the immunoaffinity cartridge (IAC), which had been washed with 20 ml of PBS solution. The sample extract was loaded onto the IAC (ca. 1–2 ml/min) using an adapter (Biocode) and a 10-ml syringe. This operation was repeated 3 times with 5-ml portions of PBS–methanol. The IAC was then washed with 10 ml of water and dried with air (syringe). The OA was eluted with 3 ml of methanol (ca. 0.5 ml/min) and air was passed through the column (syringe) to collect all the eluate in a 3-ml vial. The eluate was evaporated to dryness at 40–45°C under a stream of nitrogen and carefully dissolved in exactly 50 μl of HPLC solvent; 20 μl of this solution was analysed for OA and the remaining 30 μl were prepared for derivatization by evaporating to dryness under a stream of nitrogen. After use, the IAC was immediately washed with about 20 ml of PBS solution and stored (filled with PBS solution) at 2–8°C until the next use.

2.7. Derivatization

Methyl ester

After the addition of 2.5 ml of methanol (or another alcohol if another ester is desired) and 0.1 ml of concentrated hydrochloric acid (37%, Merck, p.a., No. 317) to the OA residue, the vial was closed and kept overnight at room temperature. After evaporating the reaction mixture to dryness and dissolving the residue in 30 μl of HPLC solvent, 20 μl of it were analysed for OA methyl ester. Based on the amount of unreacted OA the typical yield of methyl ester was estimated to be 85%.

Ochratoxin α

To the dried OA residue (see immunoaffinity cleanup) was added 100 μl of carboxypeptidase A (Sigma, Nr. C-0261, 5000 units in 4 ml aqueous suspension with toluene) and 2 ml of PBS solution. This mixture was incubated for 16 h at 38°C (nominal). A 10-ml volume of the $NaCl$–H_3PO_4 mixture (see extraction) was added to the reaction mixture and ochratoxin α was

extracted twice with chloroform (5 ml each). The chloroform extracts were evaporated to dryness at 40–50°C, following which the methyl ester of ochratoxin α was prepared as described above.

3. Results and discussion

3.1. Chromatography, detection and derivatization

Chromatography

Most methods for the analysis of OA are based on RP-HPLC with an acidic aqueous mobile phase mixed either with acetonitrile or methanol. We chose methanol because it is less toxic, less expensive and easier to degas. Tests with different types of commercially available RP columns (e.g. RP-18, ODS-2, Phenyl) demonstrated that the chromatography of OA was not tricky (in contrast to citrinin, also a nephrotoxic mycotoxin). This was confirmed by the results of a recent collaborative study, except that we also obtained good performance with ODS 1 [21]. Because of the chemical stability of silica gel-based columns we favoured the acidic system over an ion-pair technique, with the pH slightly above 7 [12,13]. The restriction of our chromatographic system is, that it is not possible to simultaneously analyse OA and citrinin [22].

The retention times (t_R) of OA and its methyl ester were not very sensitive to slight changes in the composition of the eluent. This is in contrast to the ion-pair technique at pH 7.5, in which small changes in the methanol concentration result in considerable variation in the OA retention [12]. The day-to-day variation of the retention time of OA was generally ca. 1%. Table 1 gives the relative t_Rs of some substances that could interfere in the analysis of OA.

Detection

The addition of ammonia solution to the column eluate before passing it into the detector [23], resulted, after optimizing the excitation and emission wavelengths, in about a six-fold increase of the response for OA as well as for its methyl ester. Additionally, the responses of some other coeluting peaks were markedly reduced, as has recently also been established by

Table 1
Relative retention times of OA, related substances, and some derivates in two HPLC separation systems

Compound	Relative t_R (Spherisorb ODS 1, temperature 50°C)	
	Water[a]–methanol (1:1, v/v)	Water[a]–methanol (7:18, v/v)
Ochratoxin α[b]	0.23	0.71
Ochratoxin B[c]	0.66	0.89
Ochratoxin α methyl ester	0.67	0.89
Ochratoxin A	=1.00 (19.76 min)[d]	=1.00 (5.01 min)[d]
Ochratoxin A methyl ester	2.04	1.32
Ochratoxin B methyl ester	-	1.13
Ochratoxin A ethyl ester (Ochratoxin C)	-	1.50
Ochratoxin A *n*-propyl ester	-	1.76
Ochratoxin A *n*-butyl ester	-	2.13

[a] Containing 9% (v/v) acetic acid (pH 2.3).
[b] *R* - 5 - Chloro - 3,4 - dihydro - 8 - hydroxy - 3 - methyl - 1 - oxo - 1H - 2 - benzopyran - 7 - carboxylic acid, formed from OA by enzymatic hydrolysis with carboxypeptidase A (see derivatization), which cleaves the phenylalanine group of OA.
[c] Same structure as OA, without chlorine.
[d] t_R at 1 ml/min.
–Not measured.

other authors [24]. The coefficient of variation of the peak height of OA at different injections of the same standard solution was generally 3%.

With the described system a detection limit (signal-to-noise ratio of 3) of 2 pg for OA and of 5 pg for its methyl ester was achieved. The quantitation limit for OA in blood serum or plasma (2 g) was estimated to be about 5 pg/g and that in human milk (5 g) 5–10 pg/g, depending on the type and amount of interfering peaks, including those from reagent blanks. This is somewhat better than the recently published quantitation limits for OA in cow and human milk (2 ml) of 40 pg/ml and in human blood (1 ml) of 60 pg/ml [15], but comparable to that reported by Gareis of 10 pg/ml for human milk [25]. The mean recovery of OA from human serum, milk as well as from selected foodstuffs is estimated at 85% (see below) and the overall coefficient of variation of its determination at 10% (concentration range 0.01–5 ng/g), corresponding to a "repeatability limit" of about 30%. Due to the absence of a collaborative trial and a certified standard reference material neither the reproducibility limit of the method nor the accuracy (trueness) of the results were determined.

Derivatization

Most authors use boron trifluoride as a catalyst to form the methyl ester of OA [6,12,15,26]. However, we were unable to find a commercial boron trifluorid–methanol reagent that gave an acceptable blank value (≤ 2 pg/g) although the reagents of four companies were tested. The use of mineral acids, such as conc. HCl and H_2SO_4 as catalysts is favoured by Japanese authors [13,27,28]. Because of its volatility we chose conc. HCl. The OA methyl ester was reproducibly formed with a mean yield of 82.0% and a standard deviation of 5.5% ($n = 6$) starting with standard OA concentrations of 67 and 6.7 ng/ml. If the OA concentration in a specific sample was high enough (>20 pg/g), the relative amounts of reacted and unreacted OA could be used as an additional proof of its identity, as proposed by Takeda et al. [27].

Ochratoxin α was prepared according to the protocol of Hult et al. [29,30]. The methyl ester of ochratoxin α was prepared in the same way as that of OA.

3.2. Extraction, cleanup and confirmation

Extraction

For the extraction of OA from blood serum two procedures have been established: Prior to the extraction of OA by chloroform from 2 ml of serum, (1) 10 ml of 0.1 mol/l $MgCl_2$ and 0.05 mol/l HCl (pH 2.5) is added [6,8,12,26,31], or (2) 10 ml solution of 0.5 mol/l H_3PO_4 (pH 1.6) and 2 mol/l NaCl [25,28,32].

Starting with the first protocol the following mean recoveries were achieved ($\pm$ standard deviation): $73 \pm 8\%$ ($n = 3$) for pig plasma at an OA level of 0.7 ng/g, $73 \pm 3\%$ ($n = 4$) for pig blood at OA levels of 0.2 ng/g and 0.02 ng/g, and $85 \pm 5\%$ ($n = 11$) for water at an OA concentration range of 0.02–0.7 ng/g. From cow milk, however, the recoveries obtained with this method (after a defatting step) were only about 50% at best.

By applying the second procedure (lower pH, higher ionic strength), the following OA recoveries were obtained ($\pm$ standard deviation): $86 \pm 4\%$ ($n = 4$) for cow milk at OA levels of 8–70 pg/g and $85 \pm 4\%$ ($n = 3$) for human serum at spiked OA levels of 1.0 and 2.0 ng/g. The two extraction procedures were compared using a naturally contaminated sample of pig plasma. Procedure 1 gave a mean concentration of 0.20 ± 0.02 ng/g ($n = 4$) whereas procedure 2 gave a 40% higher value of 0.29 ± 0.03 ng/g ($n = 4$). Fig. 1 gives the OA concentration of this sample (extracted 4 times instead of twice with 5-ml portions of chloroform) as a function of the NaCl concentration. Our results confirm those of Uchiyama et al. [28], who studied the extractability of OA from model solutions of bovine serum albumin and proteinous food and demonstrated that the presence of a 2 mol/l NaCl solution gives optimal results.

Cleanup

The chloroform extracts from serum or plasma are often subjected to a mini silica-gel column

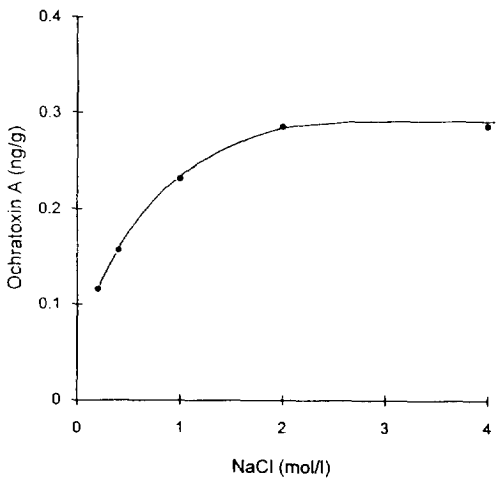

Fig. 1. Effect of sodium chloride concentration on the extractability of OA (at pH 1.6) from a naturally contaminated pig plasma sample.

cleanup, whereby OA is eluted with toluene–acetic acid (9:1, v/v) [15,31]. However, we were not able to procure a silica gel that delivered an acceptable blank value for OA (<2 pg/g), although the products of four different companies were tested. A similar experience with silica gel during analysis of pig serum was also mentioned by Unglaub and Holl [31], other authors recommended washing the silica gel with chloroform [15] or chloroform–methanol (2:1, v/v) before use [11].

In 1991 and 1992 we had the opportunity to obtain experimental IACs from Biocode. However, the first batch contained up to about 0.5 ng OA per cartridge, equivalent to an apparent serum concentration of ca. 0.3 ng/g. But after first washing the cartridges with PBS and methanol, excellent blank values and recoveries were achieved. Today the OA content of these IACs is less than 2 pg. They retain OA as well as its methyl and ethyl ester. Ochratoxin B is retained to about one third, but the methyl ester of ochratoxin α is not retained at all. Generally clean and colourless extracts were obtained for various types of samples, except if they contained haemoglobin (not adequately prepared serum samples); in this case the final extracts remained coloured.

The experimental results concerning the potential re-use of the IACs as well as the amount of serum that could be analysed by an IAC without loss of OA are summarized in Table 2. The results were obtained on a naturally contaminated human serum sample. A tendency towards decreasing OA concentrations with increasing amounts of sample, as well as with the total amount of serum to which the specific IAC

Table 2
Efficiency of the IAC as function of its load with a pooled human serum and after repeated use

Total amount of serum[a] (g)	Number of a specific IAC	Amount of serum analysed (g)	OA concentration[b] pg/g		%
0	35, 36	0.5	344 ± 4	(2)	100.0[c]
0.5	35, 36	1.0	344 ± 2	(2)	100.0
0	37, 38	2.0	309 ± 15	(4)	89.8
5–10	28	0.5	338 ± 10	(3)	98.3
	39	0.5	326 ± 13	(3)	94.8
	28	1.0	266 ± 30	(3)	77.3
	40	2.0	315 ± 23	(3)	91.6
	28	2.0	250 ± 2	(2)	73.5
20	21	2.0	336	(1)	97.7
24	21	2.0	250	(1)	72.7
	28	0.5	173 ± 42	(5)	50.3

[a] Total amount of human serum to which the IAC has been exposed by the analytical procedure before its re-use.
[b] Mean ± standard deviation, in parenthesis number of replicates.
[c] The highest mean value is taken as 100%.

has been exposed before, is recognizable. There is also a tendency to an increasing standard deviation with repeated used IACs. From the data in Table 2 it can be concluded that an IAC can be used up to about 20 times, if the amount of analysed serum does not exceeded 0.5 g per run. This is comparable on experience with self-produced IACs, which could be used more than 30 times in the cleanup of coffee extracts [33]. An alternative cleaning procedure of the used IAC could eventually increase the number of re-uses possible.

Confirmation

The OA concentrations of about one third of the human serum samples were also determined as methyl ester. The results for 81 individual serum samples (single measurements) are presented in Fig. 2 on a double logarithmic scale, demonstrating a good correlation between the two methods. Because the analyte levels should cover the measured concentration range uniformly when comparing different methods, only the concentrations less than 0.5 ng OA/g (76 samples) were taken into account to calculate the regression line ($y = a + bx$). If y is the OA concentration (ng/g) measured as methyl ester and x the OA concentration (ng/g) measured as such, the linear regression results in an intercept ($\pm$ standard error) of $a = 0.01 \pm 0.01$ and a slope $b = 0.907 \pm 0.037$ (correlation coefficient of 0.944; figure not shown). Only the slope is probably different from the theoretical value of one (t-test; $0.01 < p < 0.05$). This could be explained by some additional loss during the formation and isolation of the ester in sample extracts as compared to OA standard solutions.

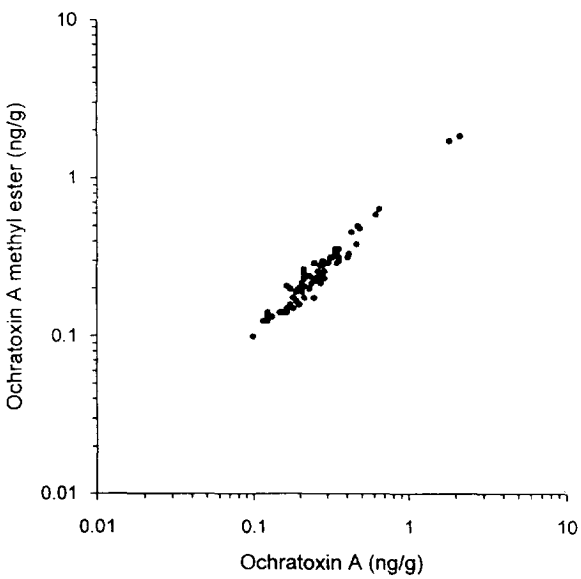

Fig. 2. Comparison of the OA results of human serum samples determined as such and as its methyl ester.

For the analytical data mentioned in the next section (applications) only the OA values obtained as such were used, and if not otherwise stated, without any correction for recoveries. The data given in the next section are generally based on single measurements.

The formation of the methyl ester of ochratoxin α may also be used for confirmation purposes (see Table 3).

4. Applications

4.1. Chromatograms

Fig. 3 shows typical chromatograms of OA and OA methyl ester from samples of naturally contaminated human milk and serum, as well as chromatograms of the reagent blanks of the procedure for the two matrices.

4.2. Human milk

Of the 40 analysed samples (north of the Alps) only one (a diabetic) had an easily measurable OA concentration of 14 ± 2 pg/g ($n = 3$), in 3 samples the concentration was estimated to 5 pg/g (overall 10% positive). The results were quantitatively confirmed by measurement of the OA methyl ester. In a German investigation of mother milk 3 out of 36 samples were positive (11%) with concentrations of 17–30 pg/g [6]. In a recent Swedish study of 40 samples, 23 contained OA (58% positive) at comparable levels of 10–40 pg/ml [15]. From Italy, however, about a hundred-fold higher concentrations of 1.7–6.6 ng/ml in 9 samples out of 50 (18% positive; detection limit 0.2 ng/ml) have been reported [34], indicating a probable higher OA body-

Table 3
Determination of OA as such, as the methyl ester, and as the methyl ester of ochratoxin α in human serum samples

Type of serum	Ochratoxin A (ng/g)	Ochratoxin A methyl ester (ng/g)	Ochratoxin α methyl ester (ng/g)
Pooled, 4 persons	0.19	0.16	0.18
Pooled, 18 persons	0.25	0.23	0.26
Individual	0.30	0.23	0.30
Individual	1.84	1.71	1.27
Individual	2.14	1.84	1.85

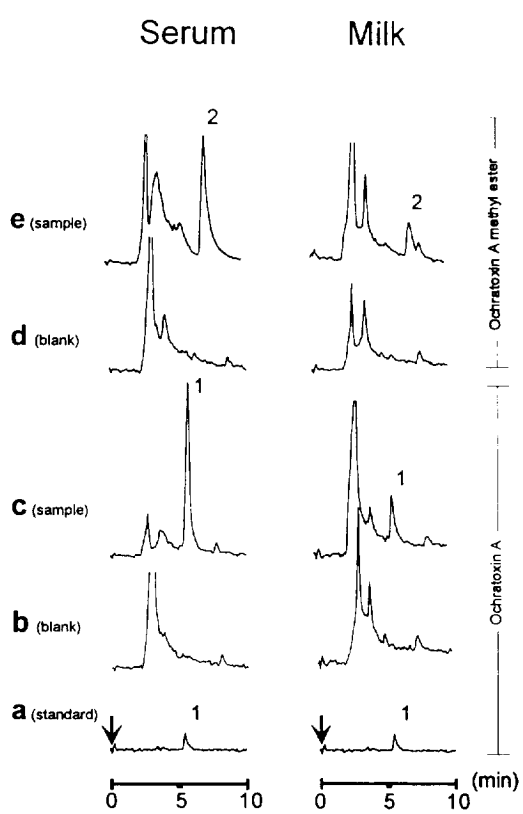

Fig. 3. Chromatograms of OA and its methyl ester in extracts of naturally contaminated human serum (equivalent to an injected amount of 0.4 g) and milk (2 g) containing 0.24 ng/g and 14 pg/g, respectively, as well as those of the corresponding reagent blanks: 11 pg OA standard (a), reagent blank (b), sample (c), with methanol esterified reagent blank (d) and corresponding sample extract (e). Peaks: 1 = ochratoxin A, 2 = methyl ester of ochratoxin A. *y*-Axis in arbitrary units.

burden than in Germany, Sweden or Switzerland.

Although in the Swedish study the OA concentrations in the blood varied by a factor of ten, no correlation between the OA concentrations in milk and blood could be detected [15]. In contrast to this, the milk:blood concentration ratios of 0.4–0.7 resulted in a short-term study with lactating rats [35]. No correlation could be established between the diet and the contamination of human milk in an Italian study [36]. Additionally, milk samples collected over six days consecutively revealed in 2 out of 6 cases peaks of OA concentrations (21.9 and 8.5 ng/ml) preceded and followed by very low levels of about 0.1 ng/ml [36].

4.3. Human serum

The separate analyses of three samples of whole human blood and serum resulted in a mean ratio of OA concentration in serum to whole blood of 2.0 ± 0.1 ($\pm$ standard error). This is in good agreement with the ratio of 1.9 ± 0.1 obtained by analysing 15 samples of pigs blood and serum. From data in the literature a somewhat higher ratio of 2.3 ± 0.2 can be estimated [37]. A corresponding ratio of 1.7 was determined for rat blood and plasma [35].

Fig. 4 shows the relative frequency distributions of the OA concentrations in the serum of men and women living on both sides of the Alps. None of these distributions correspond to a Gaussian type (probability plot), even after

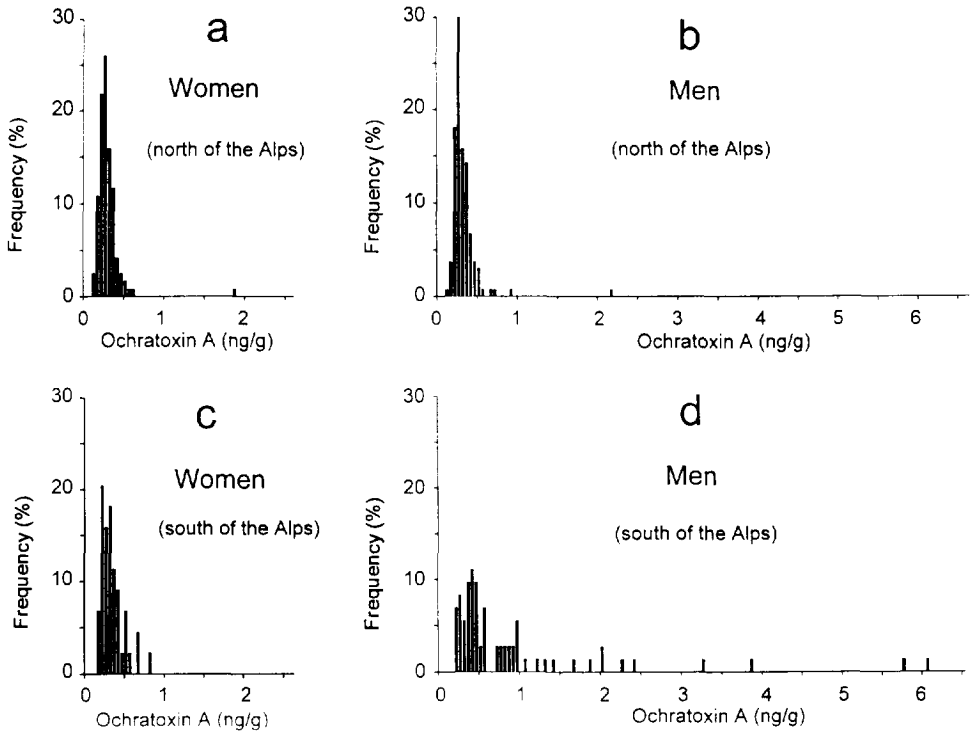

Fig. 4. Relative frequency distributions of the OA concentrations in human sera (histograms with class widths of 0.05 ng/g): Persons residing north of the Alps: (a) 119 women (median 0.23 ng/g; mean 0.26 ng/g; range 0.10–1.84 ng/g); (b) 133 men (median 0.25 ng/g; mean 0.29 ng/g; range 0.06–2.14 ng/g). Persons residing south of the Alps: (c) 44 women (median 0.29 ng/g; mean 0.30 ng/g; range 0.11–0.75 ng/g); (d) 72 men (median 0.42 ng/g; mean 0.87 ng/g; range 0.17–6.02 ng/g).

transformation (e.g. $\log x$; $1/x$; results not given), nor to a Poisson type. The overall concentrations range from 0.06 to 6.02 ng/g with medians of 0.24 ng/g ($n = 163$) for women and 0.30 ng/g ($n = 205$) for men. As far as we are aware, this is the first large-scale study of human sera resulting in 100% positive samples; this may be due to the somewhat lower quantitation limit of the present method. A German study e.g. resulted in only 56.5% positive human sera (quantitation limit 0.1 ng/ml, $n = 306$, range of positive samples 0.1–14.4 ng/g) [6]. Data from Sweden on 297 sera demonstrated significant regional differences with 13% positive sera overall (>0.3 ng/ml) and maximum values of about 7 ng/ml [12]. However, in a recent study of 39 lactating Swedish women all samples of whole blood were positive with a concentration range of 0.09–0.94 ng/ml, and a median of 0.14 ng/ml [15]. Using a factor of 2.0 for the concentration ratio of OA in the serum to whole blood results in a median of 0.28 ng/ml (range 0.18–1.88 ng/ml), which is comparable to our results for women (Fig. 4), indicating a probable similar mean daily OA intake. However, the prevalence of OA in mother milk in this Swedish study was considerably higher than in the one presented here (see above).

The results presented in Fig. 4 clearly show that Swiss males living south of the Alps have higher OA concentrations in their sera than women and than residents from north of the Alps. Practically all serum samples with OA concentrations >1 ng/g originated from male subjects living south of the Alps. Up to now, regional differences but no such distinct sex-specific ones have been reported [12,26,38]. Contrary to our data some German results indicate a tendency towards higher OA concentrations in the serum of women as compared

to males [38]. Although the reason(s) for these differences have not yet been elucidated, it is known that the consumption habits in the north and the south of Switzerland are different, e.g. the mean percental expense per household (of about 2.5 persons) in the Italian speaking part of Switzerland (south of the Alps) is higher for rice (2×), pasta (2×), bread, cake and biscuit (1.3×), olive oil (3×), coffee (1.5×), cheese (1.4×), grapes (1.8×), and tomatoes (1.2×) than in the French and German speaking parts (north of the Alps) of the country [39]. A further characteristic is a higher consumption of (mainly imported) corn (maize) as well as of alcoholic beverages especially by men (e.g. wine) in the south than in the north. It is generally known that OA is potentially associated with cereals and derived products, beans, nuts, spices, coffee, cacao beans, malt and derived products (e.g. beer), dried fruits (e.g. figs), pork and pig meat and blood (sausages) [40–44], and, as it was recently demonstrated in Sweden, also with cow milk (estimated mean concentration of 10 pg/ml) [15]. The differences in diet may help to explain the south:north ratios of the median OA concentration in Switzerland (1.7 for males, 1.3 for women), but cannot explain the fact that males from the southern part show elevated OA concentrations in their serum as compared to women. However, there is evidence that suggest that a high consumption (0.5–1 l) of certain red wines, beers or coffee can double the Swiss mean daily intake of OA (see foodstuffs).

Nevertheless, the data in Table 4 on the occurrence of OA in human sera in other countries appear to demonstrate that the average exposure of the Swiss population to OA is moderate and probably distinctly less than in France and Germany although 100% of the serum samples contained OA; the medians and/or the portion of samples with concentrations above 1 ng/ml as well as some of the maximum values are up to about 5 times higher in these countries than in Switzerland. A remarkably greater body-burden of people living in Italy and northern Africa can be assumed. It is generally stated that rural populations are more exposed to OA than urban [1,2,12].

The serum of one person of our institute (F.S.) was analysed at four occasions (data of sampling): 0.30 ng/g (May 1993), 0.31 ng/g (November 1993), 0.31 ng/g (March 1994) and 0.31 ng/g (June 1994). This seems to indicate a remarkable regular daily intake of OA. Only few such studies are described in the literature [32,45]. Individual ratios of the highest to the lowest OA level in the serum of persons sampled at different dates are in the range of 1.3–15 (estimated from available data), whereby a ratio of 3–4 seems to be most frequent.

Ten pairs of samples of maternal (venous) and umbilical cord serum (at delivery) of mothers residing north of the Alps were analysed. The concentration range of OA in maternal serum was 0.07–1.37 ng/g and 0.13–0.84 ng/g in the fetal serum. With one exception (1.37 ng/g in maternal [Tamil] and 0.84 ng/g in fetal serum) the OA concentrations in fetal serum where higher than in the maternal one, by a mean factor of 2.0 ± 0.2 ($\pm$ standard error; $n = 9$), indicating a possible active placental transport. This is believed to be the first report about the transplacental transfer of OA in humans, although its transfer has been established in pigs, rodents and other animals [46,47]. It is interesting to note that a gradient between fetuses and their mothers also exists for phenylalanine for which mean ratios in the range of 1.2 to 1.9 have been determined [48].

4.4. Foodstuffs

Our methodology has also been successfully applied to other biological matrices. In seven pale beer samples OA concentrations in the range of 10–33 pg/ml were found (median 12 pg/ml), and two malt-based breakfast drinks contained 0.10 and 0.30 ng/g (dry matter). A German study of 28 pale and 26 strong beer samples resulted in an overall median of < 0.1 ng/ml (detection limit). OA was present in 3.6% of the pale (0.3 ng/g) and in 35% of the strong beer samples (0.35–1.53 ng/g) [44]. In 18 samples of wine (2 white, 10 red and 6 rose) the OA concentrations ranged from < 5 pg/ml (5 samples) up to 0.11 ng/ml (1 sample), with typical

Table 4
Ochratoxin A concentrations in human sera from different countries

Country	Year of sampling	Sample size	Median or mean of positive samples	Minimum–maximum (ng/ml)	Relative frequency of occurrence (%) with interval (ng/ml)			Reference
					< 0.1 ng/ml	0.1–1.0 ng/ml	> 1 ng/ml	
Germany		306	0.60	< 0.1–14.4	43.5	51.6	4.9	[6,38]
Bavaria	1977/85	84 f[a]	1.30	< 0.1–8.4	32.1	25.0	42.9	[38]
Hesse	1988	124 m[a]	1.00	< 0.1–7.2	31.5	29.8	38.7	[38]
France	1991/92							
3 regions, urban		2055	[b]	< 0.1–161.0	82.9	10.1	7.0	[26]
rural		952	–	< 0.1–130.0	78.7	15.1	6.2	[26]
Canada	1990							
Manitoba (patients)		159	1.13	< 0.1–35.3	60.4	39.6		[51]
Japan	1992							
Tokyo		18	0.07	< 0.01–0.11	83.3	16.7	0	[32]
Switzerland[c]	1992/93							
north of the Alps		119 f	0.23	0.1–1.84	0	99.2	0.8	this study
south of the Alps		133 m	0.25	0.06–2.14	0.8	98.4	0.8	
		44 f	0.29	0.11–0.75	0	100	0	
		72 m	0.42	0.17–6.02	0	80.6	19.4	
Algeria	1990/92[d]							
workers		346	2.8	< 0.1–9	34.0	9.1[e]	56.0[f]	[52]
nephropathic		84	7.4	< 0.1–46	5.0	4.5[e]	90.5[f]	[52]
Tunisia	1999/92[d]							
general public		442	3.5	< 0.1–100	18.0	82.0		[53]
nephropathic		310	56.0	2–100	0	100		[53]

[a] f = female, m = male.
[b] Not given.
[c] Adults, 20–40 years.
[d] Presumably.
[e] Up to 2.0 ng/ml.
[f] > 2 ng/ml.

concentrations of the positive samples (mainly red) in the range of 10–20 pg/ml (overall median 15 pg/ml). To our knowledge this is the first time that the presence of OA in wine has been demonstrated. All these samples were analysed according to the protocol for serum using 5 ml instead of 0.5–2 ml (recovery 82.3 ± 8.5% at 70 pg/ml; $n = 3$).

Contrary to data given in the literature [16] the analysis of brewed coffee was more tricky (interfering coeluting compounds; partly low recoveries) and the procedure is not yet optimized (method not given). To get some idea on the potential OA concentration in coffee 20 samples of various brewed coffee (5 instant, 9 home-made and 6 prepared in the laboratory) were analysed. Coffee prepared from instant powder (1 g/50 ml water) tended to contain less OA than those from roasted and milled beans (3 g coffee powder/50 ml water or commercially brewed coffee). The estimated concentrations were in the range of < 2 pg/ml (10 samples) to 65 pg/ml (1 sample), typical concentration of the positive samples were in the range of 10–20 pg/ml, overall median 15 pg/ml (figures corrected for 50% recovery).

In a freeze-dried pooled total diet sample (mean caloric energy of 9460 kJ) dating from 1984 and representing the mean of 39 daily menus (breakfast, lunch and dinner) from 4 restaurants (without snacks and additional beverages) located in Berne (north of the Alps), 0.1 ng OA/g (dry matter) was estimated, indicating a mean daily intake for men of at least 45 ng OA. The analysed sample represented a mean daily intake of about 200 g (dry matter) of cereals such as bread and pasta [49]. The method used for this complex matrix was the one for human milk (recovery 83.5 ± 4.9% at 0.5 ng/g; $n = 2$).

In Germany the mean daily OA intake for adults has been calculated to be about 70 ng, based on a daily consumption of 205 g of grain products [41]. If the consumption of wine, beer and coffee (3 dl each) as well as 0.5 l of cow milk are taken into account, this would add an additional mean intake of about 20 ng to the German estimate. Although only small amounts of OA were found in the above mentioned beverages, they could be of some importance to consumers of large quantities. Under special circumstances these concentrations could probably lead up to a doubling of the mean daily intake of OA.

Based on a mean daily intake of 50 ng OA by men of 70 kg (0.7 ng/kg b.w.) and a median serum concentration of 0.25 ng/ml (steady-state) a biological terminal half-life of OA in men of 70 days would result[1], if the apparent volume of distribution (V_β) of OA and its absorption from the gastro-intestinal tract are assumed to be 12 l, based on a study in monkey, and 60%, respectively [50]. If V_β is assumed to be only 3 l, the mean volume of plasma in men, a terminal half-life of 17 days would then result. These estimates should rather give a range for the possible half-life of OA than an exact value.

Under the assumption that a mean daily OA intake of 0.7 ng/kg b.w. corresponds to a mean steady-state concentration in the serum of 0.25 ng/ml, then a daily intake of 5 ng/kg b.w. (VSD) should result in a serum concentration of about 2 ng/ml. Thus, the data in Fig. 4 seems to suggest that about 10% of the men residing south of the Alps could occasionally have higher OA intake than the proposed VSD (3% more than threefold); for the whole Swiss population it would amount to about 3%. However, there is no epidemiological evidence that the mortality rate of occurrence by kidney and urinary tract diseases, including tumors, is higher in the southern than in the northern part of the country neither in men nor in women.

5. Conclusions

The presented method using immunoaffinity cartridges for cleanup purposes during the HPLC determination of OA in human blood, serum, milk, and total diet samples as well as in beer and wine has proved to be very specific. In over

[1] $t_{1/2} = 0.693 C_{ss} V_B I^{-1} f^{-1}$; $t_{1/2}$ = biological half-life (days), C_{ss} = steady-state concentration in plasma (ng/ml), V_B = apparent volume of distribution (ml), I = mean daily intake (ng/day), f = fraction of absorption in the gastro-intestinal tract.

100 cases in which the results obtained as such have been quantitatively confirmed by forming the methyl ester of OA, no false positive or negative results have been detected. The improved quantitation limit for OA in these matrices is in the order of 5–10 pg/g.

The results appear to demonstrate that the average exposure of the Swiss population to OA is less than in adjacent countries. Furthermore, it seems that only about 3% of the Swiss population could occasionally have an OA intake exceeding the proposed VSD. This appears to indicate that in Switzerland at present OA should not be a matter of serious concern. Nevertheless, the exposure of the population to OA, a potential human carcinogen, should be as low as reasonable achievable.

This study clearly demonstrate that as the detection limit of analytical methods improve, OA can be detected in all Swiss human blood samples as well as in many different kinds of foodstuffs. Probably the number of countries where OA is present as an ubiquitous contaminant in human blood is much more frequent than commonly reported in the literature.

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