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Short communication

Determination of ochratoxin A in bile of swine by high-performance liquid chromatography

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Abstract

A method for the determination of low concentrations of the mycotoxin ochratoxin A (OA) in bile of pigs is described. OA was extracted with acidified chloroform and the extract was purified on a silicagel cartridge, followed by liquid–liquid partition. OA was determined by high-performance liquid chromatography with fluorescence detection. The detection limit was 0.3 ng/ml, the mean recovery was 84%. An enzyme-linked immunosorbent assay proved to be suitable for confirmation of positive results. Because of the low detection limit, biliary elimination of OA in pigs fed with low-contaminated feedstuffs can be examined by this method. This is an important requirement for the study of pharmacokinetic profiles of OA.

1. Introduction

Natural occurrence of the nephrotoxic, teratogenic, carcinogenic and immunosuppresive mycotoxin ochratoxin A (OA) in food and feedstuffs is widespread [1]. Transfer of OA from contaminated feedstuffs to various compartments in pigs and corresponding residues of the toxin in food products from slaughter pigs have been described by several authors [2–5].

To find out methods suitable for reducing OA residues in contaminated pigs, basic knowledge of OA elimination is important. Up to now, OA elimination primarily was studied in mice or rats after application of high OA doses [6–10]. In pigs and other livestock animals only few results limited to measurements of renal and faecal elimination were published [11–14]. Because of the enterohepatic circulation [7] and microbial degradation of OA in the intestine [15], determination of OA in bile is more adequate for studying biliary elimination than determination in faeces.

For the determination of OA in bile after application of high doses low-sensitive thin-layer chromatography methods were employed in most cases [10,14,16]. Bile extracts were applied to the plates directly without clean-up. Fuchs et al. [17] used a spectrophotometric method, Sova et al. [18] radioimmunoassay.

The aim of this study was to develop a sensitive method for the determination of OA in bile, which would make it possible to examine biliary elimination in pigs fed with feedstuffs contaminated to the low degree naturally occurring in Germany. The method consists of extraction and

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clean-up by liquid–liquid partition corresponding to a modified procedure often used for blood serum and kidneys [4], and an additional clean-up step by solid-phase extraction. OA was determined by high-performance liquid chromatography (HPLC) with fluorescence detection.

2. Experimental

2.1. Reagents

OA was purchased from Makor Chemicals (Jerusalem, Israel). OA-antiserum and OA-enzyme conjugate for the enzyme-linked immunosorbent assay (ELISA) confirmation test were obtained from the Institut für Hygiene und Technologie der Milch, Universität München (Munich, Germany). β-Glucuronidase, type H-1 (activity 300 000–400 000 U/g, sulphatase activity 15 000–40 000 U/g) from Sigma (Deisenhofen, Germany) was used for enzymatic hydrolysis. Acetonitrile was of HPLC grade, all other chemicals were of analytical-reagent grade. Silica cartridges (Adsorbex Si 400) were obtained from Merck (Darmstadt, Germany).

2.2. Bile samples

Naturally OA-contaminated samples were obtained from a feeding experiment with growing pigs with low-contaminated feedstuff (100 μg/kg); bile was collected from the gall-bladder after slaughtering. For recovery experiments, bile from pigs from a slaughterhouse was used.

2.3. Apparatus

The HPLC equipment consisted of a pump (Type 420), an autosampler (Type 360), a fluorescence spectrophotometer (SFM 25) and a data system (450-MT), all of Kontron (Neufahrn, Germany), and a combination of a SuperPac Spherisorb ODS-2 Cartridge (250 mm × 4 mm I.D., 5 μm) with an ODS-2 guard column (10 mm × 4 mm I.D., 5 μm), both from Pharmacia Biosystems (Freiburg, Germany).

For the ELISA tests, an MR 5000 microplate reader, an Ultrawash II microplate washer, both from Dynatec (Denkendorf, Germany), and Immunoplate II microtitre plates from NUNC (Wiesbaden, Germany) were used.

2.4. Extraction and clean-up of bile samples

The extraction procedure is a modification of the method of Bauer and Gareis for blood serum [4]. A 3-ml portion of bile was mixed with 20 ml of 0.4 *M* magnesium chloride solution and acidified to pH 1.6–2.0 with 2 *M* hydrochloric acid. After addition of 15 ml chloroform, the flask was shaken automatically for 30 min and then centrifuged at 1700 *g* for 25 min.

For the clean-up, a 10-ml aliquot of the chloroform extract was run through a silica cartridge, prewetted with 6 ml of chloroform; the eluate was collected, as it contained some OA. The remainder of the OA was eluted with 10 ml of chloroform–formic acid (133:2, v/v). The combined eluates were extracted twice with 10 ml of 0.4 *M* sodium hydrogencarbonate solution (the solution was added dropwise because of foaming); for clarification, the tube was centrifuged at 1200 *g* for 10 min. The combined aqueous layers were acidified with concentrated formic acid to pH 1.6–2.0, and re-extracted twice with 4 ml of chloroform. The combined chloroform extracts were evaporated to dryness on a rotary evaporator and the residue was dissolved in 0.5 ml of methanol.

2.5. Enzymatic hydrolysis of bile for the detection of conjugated OA

The bile samples were hydrolyzed as described for urine by Valenta et al. [19]: 1 ml bile, 0.3 ml 0.2 *M* sodium sulphate solution, 0.4 ml acetate buffer (pH 5.5) and 0.3 ml β-glucuronidase solution (5 mg/ml in 0.2 *M* acetate buffer) were mixed and incubated overnight at 37°C. The incubated samples were extracted according to the procedure above, but only 10 ml of the magnesium chloride solution were added and the mixture was acidified with concentrated hydrochloric acid.

2.6. Chromatographic conditions

As a mobile phase acetonitrile–0.008 *M* orthophosphoric acid (56:44, v/v), adjusted to pH 2.8 with orthophosphoric acid, was used. The flow-rate was 1 ml/min. The excitation wavelength was set at 330 nm and the emission wavelength at 460 nm. A 20-μl volume of the extract was injected. Calibration curves were based on the results from analysis of OA standard solutions in methanol in the range of 1–100 ng/ml and on spiking bile samples at different concentrations from 1 ng/ml to 20 ng/ml. The calibration plot was linear in both cases.

2.7. Confirmation by ELISA

The determination by ELISA was performed according to the method of Märtlbauer [20] for blood serum and kidneys. A defined volume of the HPLC extract was evaporated to dryness and the residue was dissolved in 0.13 *M* sodium hydrogencarbonate solution (to a final OA concentration in the range 0.05–1.00 ng/ml).

3. Results and discussion

First tests with a simple clean-up method consisting only of liquid–liquid partition as described above, which is used widely for blood serum and kidneys [4,3], did not prove sufficient for bile. No interference of the OA peak with background peaks occurred, but the recovery was low, amounting to ca. 50%. Addition of bile extract, which was purified only by liquid–liquid partition, to a standard solution of OA decreased the fluorescence signal. Therefore, bile pigments in the extract probably weakened the fluorescence and led to low recoveries. The use of recently published methods for detection of OA in urine and faeces [19] for bile did not result in better recoveries.

The interfering matrix substances could be eliminated by adsorption on silicagel. Because OA did not adsorb completely on silicagel, the eluate was collected. The adsorbed part of OA was eluted with a mixture of chloroform and formic acid. The use of solid-phase extraction also avoided emulsions, which otherwise occurred during the following liquid–liquid extraction of the chloroform phase with sodium hydrogencarbonate solution.

In Fig. 1, chromatograms of (a) a blank bile sample and (b) a sample spiked to contain 10 ng/ml OA are presented. As can be seen, there are no interfering peaks. The detection limit of the method was 0.3 ng/ml (signal-to-noise ratio 3:1). The average recovery was 84% in the range 1–17 ng/ml. The results of the recovery experiments are detailed in Table 1. The within-day and between-day coefficients of variation for a bile sample spiked to contain 10 ng/ml (analysis in duplicate on six different days) were found to be 5.8% and 9.4%, respectively.

Because Roth et al. [7] found OA–glucuronide conjugates in the bile of mice, a test was carried out for the occurrence of glucuronide conjugates in the bile of pigs with three naturally contaminated bile samples. The samples from a feeding experiment with low-contaminated feedstuffs were analysed alternatively with and without an enzymatic incubation with β-glucuronidase before extraction. The sample preparation procedure proved also suitable for the incubated samples. No additional background peaks appeared in the chromatogram after enzymatic hydrolysis. In all three samples higher concentrations of OA were measured after the enzymatic incubation. The concentrations with/without enzymatic incubation were: 15.8/11.6 ng/ml, 18.0/14.9 ng/ml and 0.7/0.3 ng/ml, respectively. These results are similar to those found by Roth et al. [7]; in their study 28–68% of the total OA content were contributed by OA–glucuronide conjugates.

An ELISA test developed by Märtlbauer [20] for the determination of OA in serum and kidneys (with a cross reactivity of ochratoxin C of 44%, ochratoxin B of 14% and ochratoxin α of $<0.1\%$) proved suitable for confirmation of positive bile samples. Six bile samples from the feeding experiment described above with OA concentrations in the range 0.3–10.0 ng/ml (measured by HPLC) were checked by ELISA. In all samples OA was confirmed by ELISA.

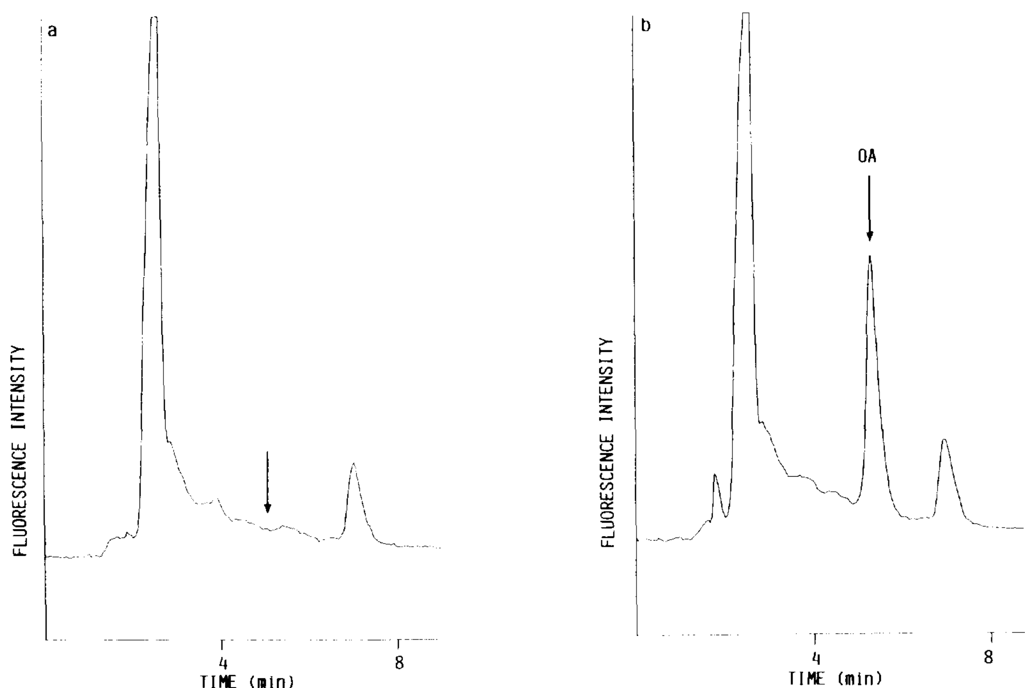

Fig. 1. HPLC of (a) a blank bile sample, and (b) a bile sample spiked to contain 10 ng/ml ochratoxin A (OA) corresponding to 0.8 ng OA injected. Column, SuperPac Spherisorb ODS-2, 5 μm (250 mm × 4 mm I.D.); mobile phase, acetonitrile–0.008 *M* orthophosphoric acid (56:44, v/v), adjusted to pH 2.8 with orthophosphoric acid; detector, spectrofluorimeter, excitation at 330 nm, emission at 460 nm.

The conformity between the results of the two methods was good; the concentrations measured by ELISA were on an average 1.3 times higher than those measured by HPLC.

The method described allows the rapid determination of low concentrations of OA in bile. Preliminary tests indicate that an enzymatic incubation before the analysis should be done when OA–glucuronide conjugates are to be considered, too. Due to the low detection limit, the method enables pharmacokinetic studies with low OA doses in the range naturally occurring in feeds.

Table 1
Recovery of ochratoxin A from bile

Concentration range of the sample (ng/ml)	Number of samples	Recovery (mean ± S.D.) (%)
1–3	13	75 ± 13
7	11	91 ± 13
10	9	90 ± 5
17	9	84 ± 5

Bile samples were spiked with an OA standard solution before the extraction.

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