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Solid-Phase Extraction and Cleanup for Liquid Chromatographic Analysis of Ochratoxin A in Pig Serum

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Natural occurrence of mycotoxins (secondary metabolites of fungi) have been found worldwide in various crops. Their ingestion, as a result of the contaminated foodstuff, is suspected to be responsible for causing some human disease, such as aflatoxin for human hepatic cancer (van Renburg 1977). Recently, much attention has been focussed on ochratoxin A, a metabolite of the genera *Aspergillus* and *Penicillium*, as a causal agent for the endemic nephropathy and urinary tumors in Balkanic areas (Yugoslavia, Roumania and Bulgaria) (Krogh 1972, Hult et al. 1982). Since the *Penicillium* species can produce the toxin even at 5° C (Harwig and Chen, 1974), the number of countries where ochratoxin A is present as a contaminant of food and feed may be much higher than in the literature.

Japan has been importing more than half its cereal for food and feed from various countries where agricultural commodities are found to be contaminated with the toxin, but there has been few reports on the contaminant problem. Since conventional analytical methods always involve laborious manipulation (solvent extraction/alkaline back-extraction followed by quantitation and confirmation), a simple and reliable method is required to monitor ochratoxin A contamination in a variety of samples. We successfully applied temperature-controlled methyl esterification of the toxin to provide two distinct peaks, free and methylated ochratoxin A on the same chromatogram. A simple extraction and cleanup by C_{18} cartridge prior to a liquid chromatographic(LC) analysis coupled with a confirmatory test is described for the determination of ochratoxin A (OA) in pig serum which has been regarded as a representative sample for monitoring ochratoxin A in the feed (Hult and Fuchs 1986).

MATERIALS AND METHODS

Ochratoxin A (Makor Chemicals, Jerusalem, Israel) 5 mg was dissolved in 100 mL of benzene/acetonitrile (98:2). HPLC-grade methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF) and acetone (Wako Pure Chemicals, Japan) and water purified by Milli-Q SP TOC (Millipore, U.S.A.) were used. A SEP-PAK C_{18} cartridge (Waters Assoc., U.S.A) was used for extraction and cleanup of OA from pig serum. A SEP-PAK

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silica cartridge (Waters Assoc., U.S.A) was used for cleanup of pig feed extract. MeOH having been dried over anhydrous Na_2SO_4 was used for methyl esterification of samples.

Pig blood in four distinct groups was obtained from a slaughter house in the central part of Japan (Hyogo Prefecture) in September and October 1990. Serum was separated and stored at -20° C until analyzed. Pig feed was obtained from two farms.

Liquid chromatography was performed using a pump LC-6AD and a fluorescence monitor RF-535 (Shimadzu Corporation, Kyoto, Japan) and a LC column STR-ODS_M (4.6 mm x 15 cm, Shimadzu Techno-Research, Inc., Kyoto, Japan). LC conditions were as follows: column temperature 45° C, flow rate 1 mL/min, mobile phase (30:15:55) THF/ACN/2% acetic acid(HOAc), injection volume 20 ul, fluorescent detection at excitation 330 nm and emission 460 nm.

A SEP-PAK C_{18} cartridge was conditioned with 10 mL of MeOH followed by 10 mL of water, and then 5 mL of 0.05M HCl containing 0.1M $MgCl_2$ ($HCl/MgCl_2$). A 1-mL aliquot of serum was mixed with 5 mL of $HCl/MgCl_2$ and put into a 10-mL syringe connected with the cartridge. The sample solution was passed through at a slow, dropwise rate and the cartridge was washed with 10 mL of water, and then 2.5 mL of 20% THF. The toxin was eluted from the cartridge with 3 mL of MeOH into a 10 mL-tube. The cartridge was reconditioned with MeOH and water for reuse at the next step.

A fresh esterification reagent, prepared by mixing 0.2 mL of H_2SO_4 and 10 mL of MeOH, was cooled to room temperature. The eluate from the cartridge was evaporated to dryness at 40° C under reduced pressure. The residuum was dissolved in 1 mL of the reagent, then placed in a water bath (15° C, COOLNICS Model CTR-120, Komatsu-Yamato, Japan) for 30 min. After addition of 10 mL of water, the mixture was passed through the same cartridge used for the extraction, and the cartridge was washed with 10 mL of water. OA and its methyl ester (OA-Me) were eluted with 3 mL of MeOH. The eluate was evaporated to dryness. Just before injection into the column, the residuum was dissolved in 200 ul of the mobile phase and centrifuged at 15,000 rpm for 2 min. Fluorescent peak height of OA was used for quantitation, and the ratio of OA/OA-Me was used for confirmation of ochratoxin A. Authentic OA samples were also run with each sample set of the serum.

Pig feed (10 g) was extracted twice for 30 min with constant shaking in a 100-mL separatory funnel with 50 mL of ethyl acetate, 20 mL of water saturated with KCl, and 1 mL of 20% HCl (Nakazato et al. 1984). The combined organic layer was back-extracted twice with 30 mL of 2% $NaHCO_3$. The alkaline layer was washed with 50 mL of isooctane, and then acidified with 2 mL of 20% HCl. The toxin was finally extracted twice with 30 mL of chloroform ($CHCl_3$). The extract was evaporated to ca. 10 mL and put into a SEP-PAK silica cartridge and eluted with 10 mL of (9:1) $CHCl_3$/HOAc. After being evaporated to dryness, the residuum was dissolved in 1 mL of the mobile phase, and 20 ul was injected for LC.

RESULTS AND DISCUSSION

A preliminary LC analysis with 50% ACN in 2% HOAc as the mobile phase showed that the C_{18} cartridge had completely extracted the toxin (30ng) in 3 mL of serum, and 10% ACN was found to be effective as a washing solution for removal of fluorescent interference materials. The use of the methyl ester derivative for confirmation of OA identity in chromatographic systems has become a standard procedure which involves the disappearance of OA peak and subsequent shift of the retention time to that of the methylated toxin (OA-Me) through the ester formation. This procedure, however, had some disadvantages: it required precollection of the the toxin-containing fraction by LC or TLC (Hult et al. 1982, Marquardt et al. 1988), or an additional chloroform extraction after derivatisation (Terada et al. 1986), and a duplicate injection of methylated sample for LC. We considered such laborious manipulation as unnecessary, if the derivatisation reaction could be correctly controlled. Because the esterification carefully terminated prior to completion would give a mixture of OA and OA-Me with a certain ratio, the two peaks would result on the same chromatogram. This methodology was expected to afford an easy and time-saving confirmatory test.

Authentic standard (10 ng) was methylated for 30 min at 15, 20 and 25° C. These temperatures are much lower than those used by Terada et al. (1986), but a precise regulation of the reaction had been difficult under higher temperatures with shorter periods. LC analysis of the reaction mixture gave OA peak at a retention time 6.7 min and the methyl ester (OA-Me) at retention time 14.6 min with peak height ratio (OA-Me/OA) of 0.3 at 15° C (CV 6.9%), 0.5 at 20° C (CV 2.9%) and 1.1 at 25° C (CV 3.7%). Conversion rate of OA to the methyl ester was 46.2, 62.8 and 73.8%, respectively (Table 1). These results showed that the derivatisation can be precisely controlled in such a lower temperature range and affords desirable peak height ratio by regulating the temperature. The peak height was used for the quantitation of OA and the ratio (OA-Me/OA) for the confirmation of OA identity. As OA-Me gave chromatographically lower peak height than that of equivalents of OA did, actual serum samples were methylated at 15° C for the required sensitivity. The calibration curves were linear over a range

Table 1. Effect of incubation temperature on esterification of ochratoxin A

Temperature (° C)	Percent Conversion Mean ± S.D.	CV%	Peak height ratio(OA-Me/OA) Mean ± S.D.	CV%
15 ± 0.1	46.2 ±1.0	2.2	0.29 ± 0.02	6.0
20 ± 0.1	62.8 ±5.7	9.1	0.52 ± 0.02	2.9
25 ± 0.1	73.8 ±0.9	1.2	1.08 ± 0.04	3.7

Values are the mean of five determinations. S.D. denotes standard deviation and CV% denotes the coefficient of variation. LC mobile phase: 50% ACN in 2% HOAc at a flow rate 1 mL/min at 40° C. Fluorescent detection: excitation 330 nm, emission 460 nm.

of 0.25 to 10 ng/ml at incubation temperature 15° C.

The proposed method was applied to the determination of OA contamination of pig serum. The derivatisation of serum extract, however, gave a large interfering peak just before the OA-Me peak in some samples (Figure 1A). The chromatogram showed that 10% ACN was not effective in eliminating the interferences. Also the mobile phase (50% ACN in 2% HOAc) appeared not to be suitable for the analysis of samples with a very small amount of OA, since OA had a retention time in an area where there were a lot of minor flurorescent interferences. Therefore, we tried again to remove the interferences by cartridge cleanup prior to LC and to improve the mobile phase for much better resolution. Some organic solvents (THF, MeOH, ACN and acetone) were tested with varying the concentration. Among the solvents tested, 2.5 mL of 20% THF as a washing solvent and THF/ACN/2%HOAc (30:15:55) as a mobile phase were found to be the most effective and to give a cleaner chromatogram with a baseline separation between the corresponding peaks and permitted detection of both OA and OA-Me at lower levels (Figure 1B).

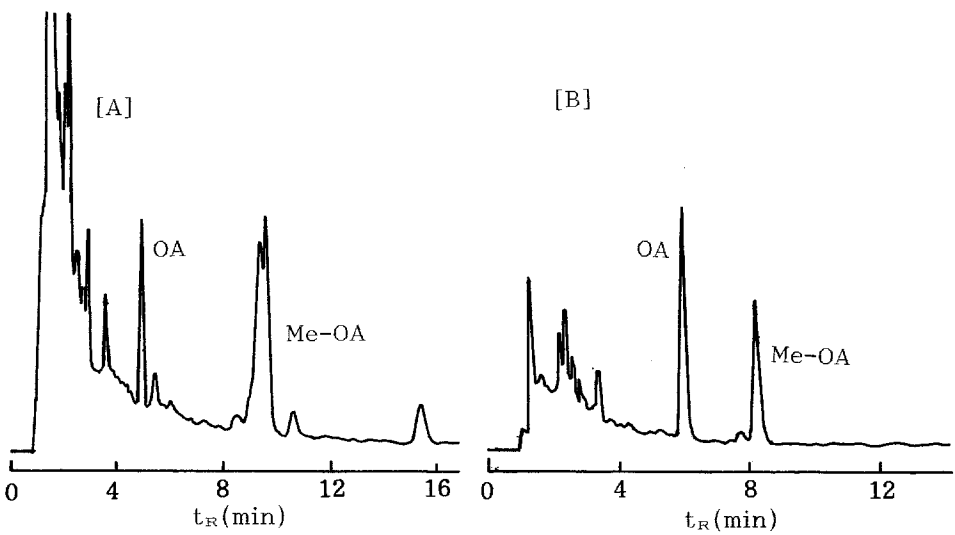

Figure 1. Chromatograms of natural occurrence of ochratoxin A in pig serum, illustrating confirmatory test and effects of washing agent and mobile phase (1 mL/min at 45° C).
[A]washing: 10% ACN 5 mL, mobile phase: (1:1) ACN/2%HOAc.
[B]washing: 20% THF 2.5 mL, mobile phase: (30:15:55) THF/ACN/2%HOAc.

With a detection limit of 0.1 ng/mL, 88% of 26 serum samples of slaughtered pigs randomly collected from four herds during the summer and early autumn of 1990 was shown to be positive for OA with a range of 0.10 - 21.6 ng/mL (Table 2). As the peak height ratio of positive samples (0.59 ± 0.08, n=24) coincided with that of authentic standard (0.6, n=4), the fluorescent peak with the retention time of 6 min was considered to be ochratoxin A. Kawamura et al. (1990) showed that all of the 19 pig sera had a mean concentration of 0.41 ppb with a

detection limit of 0.1 ppb. The higher incidence of pig serum contamination with OA in Japan compared to those in European countries and Canada (Hult and Fuchs 1986, Marquardt et al. 1988) may be due to the lower detection limit reported here.

Table 2. Ochratoxin level in sera of slaughter pigs and in feeds

Farms	Sampling Data	No. of Samples	No. of Positive Samples	Range (ng/mL)	Mean±S.D.	Feed (ug/Kg)
A	Sep. 7	4	4	0.27- 0.48	0.41±0.08	0.5
B	Sep. 7	4	4	0.13- 0.19	0.15±0.03	-
C	Sep. 7	7	4	0.10- 0.18	0.13±0.03	-
D	Oct.17	11	11	8.76- 21.6	13.5±4.4	15.2

Significantly higher OA level was detected in the serum from Farm-A compared to those from Farms B and C, however, that from Farm-D showed one or two order higher of magnitude than others (Table 2). The variation of OA contamination level in serum was small within the herd. Only two lots of commercial pig feed were available for the analysis, but the feed from Farm-D detected 30 times higher OA level than that from Farm-A (Table 2). OA levels in serum and in feed were roughly in the ratio of 1:1 in both the farms. These results support the conclusion of Hult and Fuchs (1986) that OA level in pig blood is rather homogeneous within the same herd and reflect the concentration of the toxin in the feed.

A very recent report demonstrated that 25% of 143 human sera in Czechoslovakia contained ochratoxin A at level exceeding 0.1 ng/mL, where a level of food contamination with the toxin had been found low (Fukal and Reisnerova 1990) and trace amounts of OA have been found in human milk in Germany (Gareis et al. 1988). The mycotoxic hypothesis for the etiology of Balkan endemic nephropathy begins to be more and more accepted (Hult and Fuchs 1986). A monitoring of ochratoxin A level in human and in food should be continued for elucidating the effect of prolonged and low level exposure to human health.

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