Confirmation of Ochratoxins in Biological Samples by Conversion into Methyl Esters in Acidified Methanol

Suzhen Li, Ronald R. Marquardt,* and Andrew A. Frohlich

Department of Animal Science, Faculty of Animal Science and Food Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

There is need to confirm the presence of ochratoxin A (OA) and its metabolites in biological samples following chromatographic analysis. One method is to convert the ochratoxins into different forms. A simplified procedure has been developed for the esterification of OA, OB (dechlorinated OA), Oα (hydrolyzed OA), and OA-OH (hydroxy-OA) in the presence of methanol and HCl. The percentage conversion of OA into its corresponding methyl ester (OA-Me) was >95% when the sample was incubated for a period of >12 h at 20 °C in the presence of 6 N or higher concentrations of HCl and a high relative volume of methanol (95% v/v). Similar results were obtained with OA, OB, and OA-OH when present as pure preparations or in tissue extracts of kidney, liver, bile, and blood. The conversion of Oα into Oα-Me was not quantitative. The detection limit of the assay for OA and OA-Me was 1 ng/mL. The procedure can be used for the confirmation of the ochratoxins in biological samples or for the synthesis of their esters.

Keywords: *Ochratoxins; confirmation; methyl ester; biological samples*

INTRODUCTION

Ochratoxin A (OA) is a secondary metabolite of two species of fungi, *Penicillium verrucosum* and *Aspergillus ochraceus*. It contains an isocoumarin moiety linked to phenylalanine (de Scott, 1965). The family of ochratoxins includes several structurally related analogues such as ochratoxin B (OB) and its metabolites such as the methyl and ethyl esters of OA (OA-Me and OA-Et), hydroxy-OA (OA-OH), and ochratoxin α (Oα) (van der Merwe et al., 1965; Steyn and Holzapfel, 1967a,b; Nesheim, 1969; Roberts and Woollven, 1970). OA, the primary toxin, is a potent carcinogen that accumulates in the kidney, liver, and blood of animals. It has been implicated in the fatal human disease Balkan endemic nephropathy (Krogh et al., 1977; Hult et al., 1982; Marquardt and Frohlich, 1992). The hydroxy form of OA and its hydrolyzed product, Oα, are nontoxic. The ethyl ester form of OA occurs naturally and has also been found to be as toxic as OA (Steyn and Holzapfel, 1967b; Chu et al., 1972). There is a need to develop simple methods to confirm the presence of OA and its metabolites in tissue, especially in samples that are subjected to chromatographic procedures such as HPLC. One of the best methods is to combine HPLC with mass spectrometry (MS). HPLC/MS procedures are considered to provide unambiguous confirmation of the presence of OA and its metabolites (Cole and Cox, 1981; Marquardt et al., 1988). Another procedure is to conduct NMR analysis on purified samples. Both methods require highly specialized equipment and relatively pure preparations and are therefore expensive to conduct. Also, trace amounts (<10 ng/mL) of the toxins cannot be confirmed using either procedure, especially NMR analysis. Other procedures that can be used to confirm the presence of the ochratoxins include enzyme-linked immunoasorbent assays (ELISAs) and the enzymatic conversion of OA or its metabolites into their hydrolyzed forms (Frohlich et al., 1997). The disadvantage of the ELISA is that the limits of detection are higher than those of HPLC, the assays can give false positive or negative values, and it has varying degrees of cross-reactivity with other forms of OA. The disadvantage of the enzymatic hydrolysis of the ochratoxins to their alpha forms is that the method is time-consuming and the products have much faster elution times from an HPLC column than do their parent compounds; as a result, there is a tendency for them to merge with background contaminants, which also tend to be eluted early. Neishem et al. (1973) developed a procedure for the confirmation of OA by its conversion into its ethyl ester, OC, as it is unlikely that fluorescent non-OA contaminants would coelute with both OA and its ester. The procedure developed by Neishem et al. (1973) involved the use of ethanol in the presence of boron trifluoride, which not only produced esters of OA but also resulted in the production of many other fluorescent compounds when carried out in tissue extracts (Marquardt et al., 1988). This made it difficult to confirm the presence of OA in tissue samples on the basis of the formation of OA-Et. In recent studies in our laboratory it was observed that esters of OA could be readily formed in tissue extracts when OA was incubated with concentrated acid in the presence of the different alcohols without the production of other fluorescent compounds. The objective of this study was to determine optimal conditions for the esterification of OA and its metabolites in the presence of acidified methanol and to show that this procedure provides a simple method to confirm the presence of these compounds in either pure solutions or tissue extracts. The esters were synthesized from methanol rather than ethanol as the yields were higher, and the incidence of natural occurrence of OA-Me seems to be low.

* Author to whom correspondence should be addressed [telephone (204) 474-8188; fax (204) 474-7628; e-mail rr_marquardt@umanitoba.ca].

10.1021/jf980377c CCC: $15.00
Published on Web 09/25/1998

MATERIALS AND METHODS

Chemicals and Reagents. Ochratoxins including OA, OA-OH (4*R* configuration), Oα, and OB were produced or synthesized following procedures described by Xiao et al. (1995). The ethyl (Et) and methyl (Me) ester standards of OA, OB, Oα, and OA-OH (OA-OH-Et and OA-OH-Me) were synthesized using the procedure of van der Merwe et al. (1965). Absolute ethyl alcohol was purchased from Corby Distilleries Limited, Corbyville, ON. Methyl alcohol was obtained from Mallinckrodt, ChromAR HPLC. Capillary grade GC/MS chloroform was from Burdick and Jackson (Baxter). Other reagents and chemicals were purchased from Fisher Scientific (Winnipeg, MB) or Sigma Chemical Co. (St. Louis, MO). All solvent and reagents were of analytical grade. Healthy adult female Sprague−Dawley rats weighing ∼300 g were obtained from the University of Manitoba animal colony.

HPLC Analysis. The concentration of the ochratoxins in all samples was determined by HPLC using procedures similar to those described by Xiao et al. (1996a,b), Frohlich et al. (1997), and Li et al. (1997). The HPLC system included a Waters 712 WISP sample autoinjector (Waters, Milford, MA), an LKB 2152 HPLC collector (LAB, Uppsala, Sweden), an LKB 2150 HPLC pump, an LKB 2155 HPLC column oven system, a C-18 reversed-phase column (25 cm × 4.6 mm, Waters, Novapak ODS), and an RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) set at an excitation of 330 nm and an emission of 450 nm. All HPLC analyses were performed using either an isocratic or a gradient elution profile. The isocratic mobile phase consisted of 40% water acidified to pH 2.5 with H_3PO_4 (solvent A) and a 60% mixture of methanol and 2-propanol (9+1 v/v, solvent B) set at a flow of 1.2 mL/min. The isocratic elution times of the ochratoxin standards were 2.47, 3.48, 3.67, 3.81, 3.89, 4.41, 4.45, and 5.99 min for Oα, OA-OH, OB, Oα-Me, OA-OH-Me, OB-Me, OA, and OA-Me, respectively (Figure 1A). For the gradient elution of the ochratoxins, the same solvents (A and B) were used with the flow rate set at 1.4 mL/min. The gradient system was programmed to deliver an isocratic mixture from 0 to 5 min containing 28% B, an increase of B to 45% over 7 min, an isocratic mixture containing 45% B for 6 min, followed by an increase of B to 75% over 10 min. The column was then washed with 90% B for 8 min and equilibrated with 28% B and 72% A for 8 min. The elution times (in minutes) of the ochratoxin standards (STD) when the gradient system was used were as follows: Oα, 7.56; Oα-Me, 15.86; OA-OH, 16.47; OB, 17.24; OA-OH-Me, 20.33; OA, 22.90; OB-Me, 23.45; and OA-Me, 26.21 (Figure 1B). Isocratic separation was used for samples that did not contain extracts from animal tissues (experiments 1−3 and 5), whereas gradient elution was used for those samples that were extracted from bile, liver, and kidney (experiment 4). The minimal detection limit for OA and OA-Me was 1 ng/mL. At this concentration, the standard deviation was ∼10% of the mean for replicate analysis. This limit was at least 10-fold lower than the lowest value reported in this study.

Experimental Design. A series of experiments were carried out with pure ochratoxins and ochratoxins in tissue extracts to determine the influence of different concentrations of acid and amounts of water relative to that of methanol on the production of methyl esters from different ochratoxins. The first experiment established the effect of three concentrations of HCl (0, 2, and 4 N, samples A, B, and C, respectively) in the presence of 85% methanol (v/v) on the conversion of OA into its methyl ester when incubated for 1, 3, 6, 12, 18, and 24 h (Table 1). OA in methanol (25 μL of 2.5 μg/mL) was mixed with 75 μL of water or acid and 400 μL methanol to give a final volume of 500 μL. The samples were injected onto the reversed-phase column, and the percent conversion of the OA into its methyl ester was determined.

The second experiment was designed to determine the influence of the volume of methanol relative to that of the aqueous solution and the concentrations of HCl in the incubation mixture on the formation of the methyl ester of OA when incubated for 6 or 24 h at 25 °C (Figure 2). OA in methanol

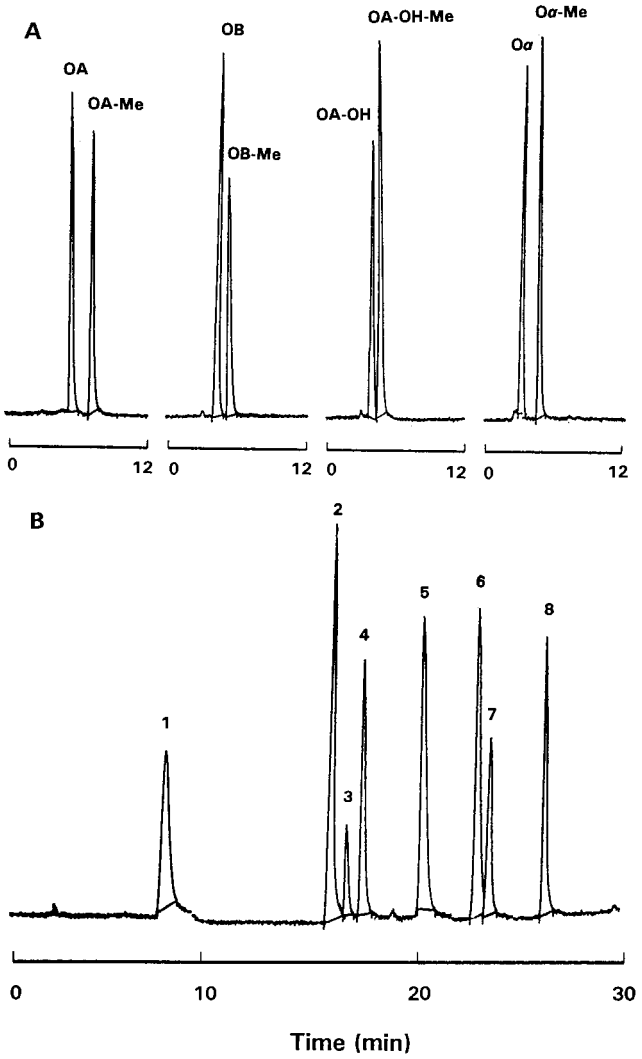

Figure 1. Chromatography of the standard ochratoxins including OA, OA-Me, OB, OB-Me, OA-OH, OA-OH-Me, Oα, and Oα-Me in short isocratic (A) and long gradient (B) elutions. The elution times for the toxins in the 12-min isocratic run were as follows: OA (4.45), OA-Me (5.99), OB (3.67), OB-Me (4.41), OA-OH (3.48), OA-OH-Me (3.89), Oα, (2.47), and Oα-Me (3.81). The gradient elution times in a 30-min gradient run were as follows: 1, Oα (7.56); 2, Oα-Me (15.86); 3, OA-OH (16.47); 4, OB (17.24); 5, OA-OH-Me (20.33); 6, OA (22.90); 7, OB-Me (23.45); and 8, OA-Me (26.21), respectively. See Materials and Methods for futher details.

(25 μL of 2.5 μg/mL) was mixed with the following volumes (microliters) of methanol (MeOH) plus water or acid (HCl): A, 475 MeOH; B, 400 MeOH, 75 H_2O; C, 400 MeOH, 50 H_2O, 25 6 N HCl; D, 450 MeOH, 25 6 N HCl; E, 450 MeOH, 25 12 N HCl; F, 450 MeOH, 25 3 N HCl; and G, 450 MeOH, 25 1 N HCl. The total amounts of acid in mixtures C and D were the same. The samples were analyzed by HPLC.

The objective of the third experiment was to determine the influence of different relative volumes of methanol (85 or 95%, v/v) and three concentrations of added acid (0, 2, and 6 N HCl) after incubation for 6 and 24 h on the formation of methyl esters from OA, OB, Oα, and OA-OH (25 μL of 2.5 μg of toxin/mL of methanol) (Table 2). The total amounts of added acid in the latter two samples were the same, whereas the final volume was 500 μL. The samples were analyzed using HPLC.

The fourth experiment involved the use of bile, liver, and kidney tissues from control and treated rats. Bile was obtained from rats administered OA or from control rats given only saline. Untreated or treated rats were injected intravenously with saline or 100 μg of OA in 1 mL of saline, respectively. The bile was collected through a bile duct

Table 1. Conversion of OA into the Methyl Ester in the Presence of Methanol Acidified with 0, 2, or 4 N HCl and Incubated for Different Time Periods (1, 3, 6, 12, 18, and 24 h) in Experiment 1

	treatment[a]		conversion of OA (%) into new product[b]					
	% MeOH (v/v)	N of HCl added	1 h	3 h	6 h	12 h	18 h	24 h
A	85	0	0	0	0	0	0	0
B	85	2	24 ± 8	48 ± 10	62 ± 8	77 ± 4	85 ± 3	87 ± 0.5
C	85	4	27 ± 4	48 ± 4	64 ± 7	80 ± 8	85 ± 3	88 ± 1

[a] The volume of water or acid added was 75 μL and that of methanol was 425 μL. The volume of OA in the final 500 μL was 25 μL (2.5 μg/mL methanol). [b] The elution time for OA was 4.45 min; that of the new product was 5.99 min, which was the same as that of the methyl ester of OA reference standard. Values represent means ± SD of three analyses.

catheter for 3−4 h after administration of the toxin. The surgical procedure and the method of extraction of OA in bile were as described by Li et al. (1997). The same procedure was used for the esterification of OA in the bile as described above except 25 μL of bile was used instead of the same amount of pure toxin in methanol. The spiked samples or control bile contained 25 μL of OA (2.5 μg of OA/mL of methanol) or 25 μL of methanol, 25 μL of control bile, or 25 μL of bile from OA-treated rats, 50 μL of H_2O, and 400 μL of methanol, respectively. The samples were incubated at 25 °C for 12 or 24 h, centrifuged for 10 min at 13000*g*, and then injected onto the reversed-phase column. The percent methanol in the sample was 85% (v/v), the final concentration of acid in the aqueous phase (bile plus acid) was 2 N HCl, and the final volume was 500 μL.

Liver and kidney samples were obtained from rats fed OA (2.5 mg of OA/kg of body weight) and injected intraperitoneally (ip) with OA (0.1 mg of OA in saline/300 g of body weight). The analysis of these samples was performed using a procedure similar to that described by Hult et al. (1982), Clarke et al. (1994), and Li et al. (1997). The kidney or liver from rats (2 g) was finely minced with scissors and placed in capped Nalgene centrifuge tubes. Ten milliliters of 0.05 N HCl/0.1 M $MgCl_2$, 6 mL of $CHCl_3$, and 100 μL of OA for the spiked samples (5 μg/mL in methanol) and 100 μL of methanol for the nonspiked samples were added into the mixtures, and they were shaken vigorously for 15 min in a mechanical shaker. The samples were then placed in an ice bath for 10 min, and they were centrifuged at 1300*g* for 10 min. Four milliliters of the chloroform layer was removed from the bottom of the tube and transferred into an opticlear tube using a syringe with a long needle. Two milliliters of distilled water was added into each tube and mixed well, and the sample was centrifuged at 1300*g* for 10 min. Three milliliters of the $CHCl_3$ layer was transferred into a glass vial and dried under a stream of N_2. The dried sample was reconstituted with 500 μL of methanol and sonicated for 2 min, and 25 μL of the sample was added to 25 μL of 6 N HCl or 25 μL of water and 450 μL of methanol. The mixture was incubated for 12 or 24 h at 25 °C. The percent methanol in the sample was 95% (v/v). The same procedure was used for extraction of the liver and kidney from rats fed or injected intraperitoneally with OA. The different preparations were then used for HPLC analysis. All of the above experiments were replicated a minimum of two times. Blood was extracted in chloroform as reported by Li et al. (1997). The N_2-dried sample was reconstituted as described for the liver and kidney. The final concentration of methanol was 95% with the amount of acid being 25 μL of 6 N acid/500 μL.

Mass Spectrometry (MS) of Acidified Ochratoxins. MS was carried out to confirm that the methyl esters were formed when OA, OB, OA-OH, and Oα were incubated in methanol and concentrated HCl (experiment 5). In a preliminary experiment, 1 mL of OA (2.5, 25, and 250 μg of OA/mL of methanol) and 1 mL of 6 or 12 N HCl were added into 18 mL of methanol and incubated for 24 or 72 h at 25 °C. Conversions of OA into its methyl ester as determined by HPLC were 96−97% in the presence of 6 N HCl and 99−100% in the presence of 12 N HCl. Therefore, for MS analysis, high concentrations of OA, OB, OA-OH, and Oα were converted into their esters using 12 N HCl for 24 h with the conversions being 99, 98, 100, and 26%, respectively. The samples (20 mL; 19 mL of methanol and 1 mL of 12 N HCl) were extracted with 20 mL of chloroform and 100 mL of distilled water in a separation funnel and shaken by hand. The chloroform layer was collected and evaporated at 50 °C under a rotary evaporator. The sample was reconstituted by adding 2 mL of chloroform and dried under a stream of nitrogen. The dried material was analyzed using a 7070 EHF VG analytical mass spectrometer (Manchester, England) in the Department of Chemistry, University of Manitoba.

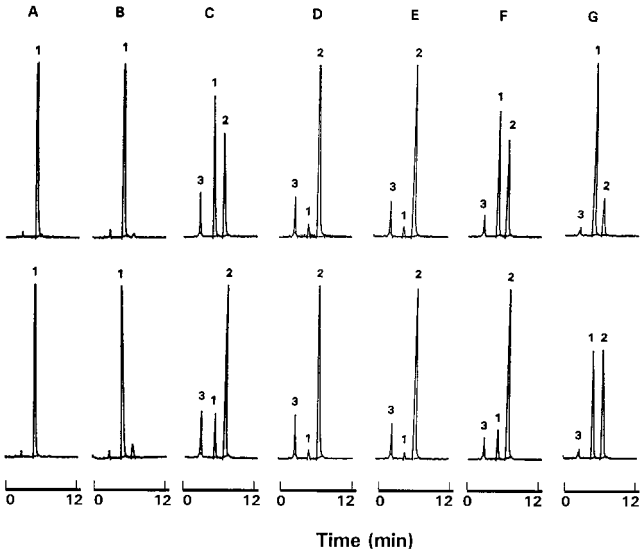

Figure 2. Chromatography of OA in methanol acidified with different concentrations of HCl and incubated for 6 (upper chromatographs) or 24 h (lower chromatographs) at 25 °C. Samples A, B, C, D, E, F, and G, respectively, contained 100, 85, 85, 95, 95, 95, and 95% methanol and 0, 0, 2, 6, 12, 3, and 1 N HCl. The volumes of added acid or water were 0, 75, 75, 25, 25, 25, and 25 μL, respectively, with the total final volume being 500 μL (see Materials and Methods, experiment 2 for further details). The elution times for peaks 1, 2, and 3, respectively, were 4.55, 5.99, and 2.47 min, which corresponded to elution times for OA, OA-Me, and Oα in the standard chromatograms (Figure 1A).

RESULTS

The results from experiment 1 demonstrated that OA, in the presence of either 2 or 4 N HCl and 85% methanol, was converted in a time-dependent manner into its methyl ester with >60% conversion being completed within 6 h and 87% at 24 h (Table 1, samples B and C). Similar results were obtained at both concentrations of acid. No OA-Me was formed in the control group without added acid (sample A). However, upon prolonged standing for more than a month traces of OA-Me ester were formed in nonacidified methanol (data not shown). In the second experiment, the volumes of methanol relative to water (Figure 2C,D), the

Table 2. Conversion of Ochratoxins (OA, OB, Oα, and OA-OH) into Their Methyl Esters When Acidified with 6 N HCl with or without Water and Incubated for 6 and 24 h at 25 °C in Experiment 3

treatment				concentration of ochratoxins (μg/mL) and conversion (%) into new product					
				6 h			24 h		
toxin[a]	% MeOH (v/v)	N of HCl added	vol of HCl added (μL)	toxins	P[b]	conversion (%)	toxins	P[b]	conversion (%)
OA	85	0	0	2.44 ± 0.02	0	0	2.43 ± 0.06	0	0
	85	2	75	0.93 ± 0.11	1.10 ± 0.87	53	0.31 ± 0.25	1.96 ± 1.32	86
	95	6	25	0.15 ± 0.05	2.36 ± 2.15	94	0.06 ± 0.10	2.41 ± 0.31	98
OB	85	0	0	2.47 ± 0.16	0	0	2.46 ± 1.39	0	0
	85	2	75	0.81 ± 0.39	0.84 ± 0.73	51	0.20 ± 0.38	1.48 ± 1.12	88
	95	6	25	0.03 ± 0.11	1.20 ± 0.17	98	0	1.99 ± 2.4	100
Oα	85	0	0	2.40 ± 1.10	0	0	2.44 ± 1.36	0	0
	85	2	75	1.49 ± 6.49	0.19 ± 0.01	11.3	1.17 ± 2.46	1.26 ± 1.12	52
	95	6	25	1.26 ± 0.24	1.16 ± 0.27	47.9	0.77 ± 0.35	1.99 ± 2.39	72
OA-OH	85	0	0	2.30 ± 2.49	0	0	2.29 ± 2.21	0	0
	85	2	75	0.95 ± 1.72	1.30 ± 3.59	58	0.24 ± 0.59	2.03 ± 1.19	89.5
	95	6	25	0.05 ± 0.3	2.25 ± 1.68	98	0.05 ± 0.11	2.32 ± 0.81	98

[a] The concentration of the stock solution for each ochratoxin was 25 μg/mL of methanol with 25 μL being added/500 μL of sample. The entire experiment was repeated twice. [b] There was no interconversion of any of the ochratoxin to other forms in the absence of acid. Incubation of the ochratoxins yields a new product (P), which in all cases had the same elution time as the methyl ester reference standard.

concentration of acid (Figure 2D−G) and the incubation time (Figure 2, upper frames, 6 h; lower frames, 24 h) affected the degree of conversion of OA into OA-Me and Oα. The degrees of conversion of OA into OA-Me were 53 and 94% after 6 h and 86 and 96% after 12 h when the concentrations of acid in the aqueous portion of the mixture were 2 and 6 N HCl and the percents methanol (v/v) in the sample were 85 and 95, respectively (C vs D). The total amounts of acid were the same in both samples. The corresponding degrees of hydrolysis of OA were 13 and 7% after 24 h and 23 and 18% after 48 h in samples containing 85 and 95% methanol. The percent conversions of OA into OA-Me in the presence of 12 (E), 6 (D), 3 (F), 1 (G), and 0 (A) N HCl and 95% methanol (v/v) were 97, 97, 60, 26, and 0% for 6 h and 98, 96, 84, 51, and 0% for 24 h, respectively.

The influence of the incubation time (6 and 24 h) and volume of methanol (85 and 95%, v/v) relative to total volume of incubation mixture in the presence of 0, 2, or 6 N HCl at 25 °C on the degree of conversion of OA-OH, Oα, and OB into their methyl esters is presented in Table 2 (experiment 3). The total amounts of HCl in the two samples containing 2 or 6 N HCl were the same. The trends were the same in all treatments, with the highest degree of formation of the methyl esters occurring in samples containing the higher relative volume of methanol and therefore a lower relative volume of water and when the incubation period was 24 h as compared to 6 h. Under these latter conditions >98% of OA, OB, and OA-OH were converted into their methyl esters, whereas the value for Oα was 72%.

In another study (experiment 4) the percent conversion of OA into its methyl ester was determined for bile, kidney, or liver samples. Kidney or liver tissues were either spiked with OA, obtained from rats fed OA, or from rats injected ip with OA. Bile was obtained from rats injected intravenously (iv) with or without OA. The chromatograms for the control samples without OA but treated with and without acid in all cases, except for bile treated with acid, did not have peaks that eluted at the same time as those of OA or OA-Me (data not shown). Acid treatment of bile, however, produced a small peak that coeluted with OA but not with OA-Me. The liver, kidney, and bile samples from OA-spiked (S) or OA-treated rats (T) had only an OA peak and no OA-Me peak (Figure 3, S and T). The same samples when incubated with 2 N (bile) or 6 N (liver or kidney) HCl and methanol [85% for bile or 95% for liver or kidney spiked (S+A) and treated kidney] had nearly quantitative conversion of OA into OA-Me except for (T+A) bile which, respectively, contained 11 and 25% OA and 89 and 75% OA-Me as determined from peak areas (Figure

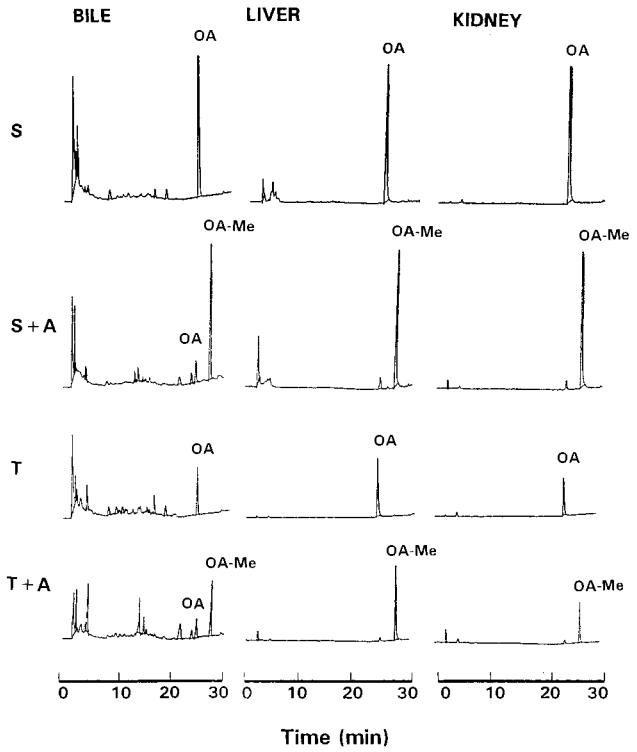

Figure 3. Chromatography of OA from tissues including bile, liver, and kidney tissue of rats acidified (S+A or T+A) or not acidified (S or T) with HCl and incubated in methanol for 12 h at 25 °C. The tissues were obtained from rats not treated with OA and spiked with OA (S) or from treated rats injected intravenously (bile) or intraperitoneally (liver and kidney) with OA (T). The percent methanol in bile was 85; that in liver and kidney was 95. The corresponding concentrations of HCl in the aqueous solution (tissue + added acid) were 2 or 6 N, with the total amounts of acid being the same for all samples. The procedure for extraction and incubation of the samples was as described in experiment 4 of Materials and Methods.

3, S+A and T+A). The apparent reduced conversion of OA to OA-Me in the two bile samples compared to the kidney and liver samples may have been caused by the presence of fluorescent contaminants that coeluted with OA in acidified bile. Also, the apparent reduced conversion of OA to OA-Me may have been caused by incomplete conversion of OA into OA-Me because the total concentration of methanol was 85% in bile compared to 95% in the other treatments. Higher conversion, under the latter conditions, could have been achieved by longer incubation periods or by the use of higher concentrations of methanol (Table 2). Although the concentrations of acid in the bile compared to in the liver and kidney samples were different (2 or 6 N HCl), the total amounts of added acid were the same (Table 2). The percent conversions of OA into OA-Me in liver and kidney samples that were incubated for 12 h in 6 N HCl and 95% methanol in the spiked tissues and in rats fed and injected ip with OA were 97, 100, and 100% for kidney and 96, 100, and 100% for liver, respectively. In this study essentially the same quantitative data were obtained when samples were incubated for 24 h. The results obtained with blood were similar to those obtained with kidney and liver with the conversion of OA into OA-Me after 12 h being 100% (data not shown).

In a final experiment OA, OB, OA-OH, and Oα were incubated with 12 N HCl in the presence of 95% methanol for 24 h. The samples were extracted and analyzed using MS. The percent conversion (>98% except for Oα) and recovery as estimated from relative peak areas (>90%) after 24 and 72 h were essentially the same, indicating that 24 h is sufficient time to complete the conversion and that the toxin did not significantly decompose in the presence of strong acid and a low concentration of water into Oα and other products. These data also demonstrate that large quantities of the methyl esters of OA, OB, OA-OH, and probably even Oα can be prepared by incubating the parent compounds in the presence of 95% methanol and 5% 12 N HCl (v/v) for 24 h at 25 °C.

In all cases the MS patterns for the major peaks of OA-Me, OB-Me, OA-OH-Me, and Oα-Me were the same when the compounds were prepared using the standard procedure (van de Merwe et al., 1965) and the modified procedure as reported in this paper. MS analysis confirmed that the compounds formed in the presence of 95% methanol and concentrated HCl (6 or 12 N) were their methyl esters. The MS patterns of OA, Oα, and OA-OH and their methyl esters revealed a chloride atom in all compounds. The mass spectrum of OA-Me had three major peaks at *m*/*z* 255, 239, and 221, which agrees with OA, and a minor peak at *m*/*z* 417, which is the molecular ion. The mass spectrum of OB-Me had three major peaks at *m*/*z* 187, 205, and 221, which agrees with that of OB, and a minor peak at *m*/*z* 383 (molecular ion). The mass spectrum of OA-OH-Me had three major peaks at *m*/*z* 237, 255, and 271 and a minor peak at *m*/*z* 433 (molecular ion). The mass spectrum of Oα had four major peaks at *m*/*z* 194, 209, 223, and 238 and an additional relatively large peak at *m*/*z* 270 (molecular ion).

Studies were also carried out with ethanol rather than methanol. Similar trends were obtained except the percent conversions of the different ochratoxins to their ethyl esters were lower than those obtained with methanol. The percent conversions of OA into OA-Et in the presence of HCl and ethanol were 55 (6 N HCl + H_2O, 85% ethanol), 86 (6 N HCl, 95% ethanol), and 88 (12 N HCl, 95% ethanol) for 6 h. The corresponding values for 24 h were 83, 97, and 97%. Also, mixed esters of OA were produced in the presence of a mixture of methanol and ethanol (data not shown).

DISCUSSION

The results of this study demonstrated that OA, OB, and OA-OH can be quantitatively converted into their methyl esters in the presence of acidified methanol with the degree of conversion being influenced by time of incubation, concentration of acid, and content of methanol relative to that of water. The degree of conversion of Oα to its methyl ester, under optimal condition, was not quantitative but was as high as 72%. Maximal conversions were obtained in the presence of high concentrations of acid (6 and 12 N) and high relative volumes of methanol (95%). Presumably, an equilibrium exists between the ester form and the nonester forms which is influenced by the relative volumes of methanol and water in solution. Also, the degree of hydrolysis of OA to Oα is probably influenced by the availability of water in the mixture. It is therefore desirable to minimize the volume of water in the sample but to have sufficiently high amounts of acid to permit the rapid formation of the esters. Also, under these conditions, there was a minimal degree of hydrolysis or decomposition of OA over time. Mass spectrometry studies as indicated under Results confirm that the new compounds were the methyl esters of the different ochratoxins. Plots of the mass-to-charge ratios (*m*/*z*) of the different compounds were as given by Cole and Cox (1981) and Xiao et al. (1995, 1996a).

When the ochraxins are to be confirmed, it is recommended that all tissues including bile and serum be extracted into chloroform following the same procedure as described above. In this procedure the samples containing OA or its metabolites are evaporated to dryness and are then diluted in methanol. These samples can then be subdivided; one part can be used for regular toxin analysis and one part for confirmation of the presence of OA by conversion into its methyl ester. The sample to be analyzed for OA can be diluted with the appropriate solution and injected onto the HPLC column while the other portion of the sample to be used for the confirmation can be mixed with concentrated HCl (12 N), diluted with methanol, and incubated for 6−24 h. A suitable ratio of 12 N HCl to methanol in the sample is 1:19 (95% methanol, v/v). Under such conditions the volume of water in the sample would be minimal. This latter procedure is superior to the procedure used in the current study with bile as it tends to have lower background values and can be designed so that a minimum amount of water is added to the sample and therefore maximum esterification can occur. A disadvantage of extracting the sample with chloroform is that it requires additional steps. This procedure is also superior to the previously used procedures for the confirmation of OA (Nesheim, 1969; Nesheim et al., 1973; Hult et al., 1982). The latter procedure involves the derivatization of OA with methanol in the presence of boron trifluoride; a procedure that does not lend itself to direct HPLC analysis as a cleanup step is required. Also, when biological samples such as blood are treated with boron trifluoride in the presence of methanol, many

new fluorescent peaks are formed, which greatly increases background interference and makes confirmation ambiguous (Marquardt et al., 1988). The present method does not generate new fluorescent peaks in biological samples and therefore avoids the problems associated with the previously used method.

The method reported in this study can also be used for the synthesis of the methyl and the ethyl ester forms of OA, OB, and OA-OH for uses as reference standards or other purposes. As indicated above, ethanol can replace methanol if problems occur with coeluting compounds or if two forms of OA are needed for confirmation. The procedure can be readily incorporated into routine HPLC analysis of the ochratoxins with a minimal amount of additional preparation time. The procedure is simple and quantitative for OA and OB. This new procedure, therefore, can be used to confirm the presence of ochratoxins in biological samples and for the preparation of the methyl and ethyl ester forms of OA, OB, OA-OH, and Oα.

ABBREVIATIONS USED

OA, ochratoxin A; OB, ochratoxin B; Oα, ochratoxin α; OA-OH, hydroxyochratoxin A; OA-Me, methyl ester of ochratoxin A; OC or OA-Et, ethyl ester of ochratoxin A; P, product.

LITERATURE CITED

Chu, F. S.; Noh, I.; Chang, C. C. Structural requirements for ochratoxin intoxication. *Life Sci.* **1972**, *11*, 503−508.

Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A.; Pitura, R. J. Quantification of ochratoxin A in swine kidneys by enzyme-linked immunosorbent assay using a simplified sample preparation procedure. *J. Food Prot.* **1994**, *57*, 991−995.

Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites: Ochratoxins*; Acdemic Press: London, 1981; pp 128−151.

de Scott, B. Toxigenic fungi isolated cereal and legume products. *Mycopathol. Mycol. Appl.* **1965**, *25*, 213−222.

Frohlich, A. A.; Marquardt, R. R.; Clarke, J. R. Enzymatic and immunological approaches for quantitation and confirmation of ochratoxin A in swine kidneys. *J. Food Prot.* **1997**, *60*, 172−176.

Hult, K.; Plestina, R.; Habazin-Novak, V.; Radic, B.; Ceovic, S. Ochratoxin A in human blood and Balkan endemic nephropathy. *Arch. Toxicol.* **1982**, *51*, 313−321.

Krogh, P.; Hald, B.; Plestina, R.; Ceovic, S. Balkan (endemic) nephropathy and foodborn ochratoxin A: Preliminary results of a survey of foodstuffs. *Acta Pathol. Microbiol. Scand. Sect. B.* **1977**, *85*, 238−240.

Li, S.; Marquardt, R. R.; Frohlich, A. A.; Vitti, T. G.; Crow, G. Pharmacokinetics of ochratoxin A and its metabolites in rats. *Toxicol. Appl. Pharmacol.* **1997**, *144*, 82−90.

Marquardt, R. R.; Frohlich, A. A. A review of recent advances in understanding ochratoxicosis. *J. Anim. Sci.* **1992**, *70*, 3968−3988.

Marquardt, R. R.; Frohlich, A. A.; Sreemannarayana, O.; Abramson, D.; Bernatsky, A. Ochratoxin A in blood from slaughter pigs in Western Canada. *Can. J. Vet. Res.* **1988**, *52*, 186−190.

Nesheim, S. Isolation and purification of ochratoxins A and B and preparation of their methyl and ethyl esters. *J. Assoc. Off. Anal. Chem.* **1969**, *53*, 975−979.

Nesheim, S.; Hardin, N. F.; Francis, O. J., Jr.; Langham, W. S. Analysis of ochratoxin A and B and their esters in barley, using partition and thin-layer chromatography. I. Development of the method. *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 817−821.

Roberts, J. C.; Woollven, P. Study in the mycological chemistry. Synthesis of ochratoxin A, a metabolite of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.* **1970**, *C*, 278−281.

Steyn, P. S.; Holzapfel, C. W. The synthesis of ochratoxins A and B metabolites of *Aspergillus ochraceus* Wilh. *Tetrahedron* **1967a**, *23*, 4449−4461.

Steyn, P. S.; Holzapfel, C. W. The isolation of the methyl and ethyl esters of ochratoxin A and B, metabolites of *Aspergillus ochraceus* Wilh. *J. S. Afr. Chem. Inst.* **1967b**, *20*, 189−189.

van der Merwe, K. J.; Steyn, P. S.; Fourie, L. Mycotoxins. Part II. The constitution of ochratoxin A, B and C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.* **1965**, *P*, 7083−7088.

Xiao, H.; Marquardt, R. R.; Frohlich, A. A.; Ling, Z. Y. Synthesis and structural elucidation of analogs of ochratoxin A. *J. Agric. Food Chem.* **1995**, *43*, 524−530.

Xiao, H.; Marquardt, R. R.; Abramson, D.; Frohlich, A. A. Metabolites of ochratoxins in rat urine and in a culture of *Aspergillus ochraceus*. *Appl. Environ. Microbiol.* **1996a**, *62*, 648−655.

Xiao, H.; Madhyastha, S.; Marquardt, R. R.; Li, S.; Vodela, J. K.; Frohlich, A. A.; Kemmainen, B. W. Toxicity of ochratoxin A, its opened lactone form and several of its analogs: Structure−activity relationships. *Toxicol. Appl. Pharmacol.* **1996b**, *137*, 187−192.

Received for review April 13, 1998. Revised manuscript received August 11, 1998. Accepted August 13, 1998. Financial support was provided by the University of Manitoba and the National Science and Engineering Council of Canada.

JF980377C