DISPOSITION AND CONFIRMATION OF OCHRATOXINS IN RATS AND BIOLOGICAL SAMPLES

By

Suzhen Li

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements for the degree

of

Doctor of Philosophy

Department of Animal Science
University of Manitoba
Winnipeg, Manitoba

November, 1998

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

DISPOSITION AND CONFIRMATION OF OCHRATOXINS IN RATS AND BIOLOGICAL SAMPLES

BY

SUZHEN LI

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

ABSTRACT

Ochratoxin A (OA) and its analogs are a family of structurally related secondary metabolites produced by fungi, *Aspergillus ochraceus* and *Penicillium verrucosum*. The objectives of this study were to develop procedures for the confirmation of the ochratoxins and to determine their half-lives and the nature of their clearance from the body. The ochratoxins that were studied were OA, OC (ethyl ester of OA), OP-OA (newly discovered lactone opened form of OA), Oα (ochratoxin alpha, hydrolyzed OA), OA-OH (hydroxylated OA), OB (dechlorinated OA) and Oβ (hydrolyzed OB). Two procedures were developed for the confirmation of ochratoxins in biological samples. This involved either their esterification or the opening of the lactone groups. HPLC analysis of the different ochratoxins showed that more than 20 different ochratoxins and their metabolites were detected in bile and urine of treated rats. The results from the pharmacokinetic studies suggested that all of the ochratoxin analogs were distributed following a two compartment open model. The elimination half-lives of OA, OP-OA, Oα, OA-OH, OB and OC were 103 ± 16, 50.5 ± 2.8, 9.6 ± 2.3, 6 ± 0.9, 4.2 ± 1.2 and 0.6 ± 0.2 h, respectively. Total body clearance of OA, OP-OA, Oα, OA-OH and OB via the bile, urine and metabolic routes were 3.1, 3.6, 40, 65 and 43 ml/h kg, respectively. OA, OB and Oα were mainly cleared in the urine (≥48%). OA-OH in the bile (41%) and OP-OA mainly as metabolites (43%). Metabolism accounted for 43, 44, 33 and 29% of the total clearance of OA, Oα, OA-OH and

OB, respectively. The results show that OA has a long half-life and is very slowly cleared from the body and that its metabolites are cleared at a much faster rate with much shorter half-lives. This research resulted in the development of two procedures for the confirmation of different forms of OA, demonstrated that many metabolites of OA are produced in the rat, established the half-lives of several of the ochratoxins, most for the first time, and provided new information on the clearance of ochratoxins from the body.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Ronald R. Marquardt, for his extensive knowledge, enthusiasm, rigorous scientific approach, devotion to the cause of science, and his encouragement to work hard. Special thanks to Dr. Andrew Frohlich for his valuable technical expertise, pragmatism and friendship. I would like to thank all members of my graduate committee, especially Dr. G. T. Vitti for his pharmacokinetic knowledge and kind support, Dr. G. Crow for his patient help with data analysis and Dr. D. Abramson for his valuable advice.

I would like to thank Dr. Loreen Onischuk for assistance in the drawing of the chemical structures and advice on computer techniques. The fellowship and friendship of Dr. Zhibo Gan, Mr. Lin Fang, Ms's. Qunying Zhang and Helena Stelsovsky and their assistance in the care of animals and with the general laboratory materials support was greatly appreciated.

Special thanks to Mrs's Allison Cranmer, Shirley McFaren and Kae Harasym for their kind and generous support in the reading and formatting of this thesis.

I would like to thank all of the people in the Animal Science building, especially the Professors. Staff and Students, for providing me with a home away from home.

I gratefully acknowledge the financial support provided by The Natural Science and Engineering Research Council of Canada.

Finally, I would like to thank to my husband, Dr. Zhengkang Han, my parents, and my brother and sister for their understanding, encouragement and support.

FOREWORD

This thesis is written in manuscript style. The first manuscript is in press in the "Journal of Agricultural and Food Chemistry", the second manuscript has been submitted for publication in the "Food and Chemical Toxicology" and the third manuscript has been published in "Toxicology and Applied Pharmacology".

The authors of the manuscripts are:

I. S. Li, R. R. Marquardt, and A. A. Frohlich

II. S. Li, R. R. Marquardt, and A. A. Frohlich

III. S. Li, R. R. Marquardt, A. A. Frohlich, G. T. Vitti and G. Crow

Additional manuscripts which are related to the research in this thesis will be submitted.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CHA= cholestyramine;

EPR= electron paramagnetic resonance;

LD_{50}= median lethal dose;

HPLC= high performance liquid chromatography;

i.d.= inside diameter;

i.p.= intra-peritoneal injection;

i.v.= intravenous injection;

MS= mass spectrometry;

OA = ochratoxin A;

OA-Me = methyl ester of ochratoxin A;

OA-OH = hydroxyochratoxin A;

OA-OH-Me= methyl ester of OA-OH;

Oα = ochratoxin α;

Oα-Me= methyl ester of Oα;

OB = ochratoxin B;

OB-Me= methyl ester of OB;

Oβ=ochratoxin β;

OC or OA-Et = ethyl ester of ochratoxin A;

o.d.=outside diameter;

OP-OA=open form of ochratoxin A;

OP-1= open form one of ochratoxin;

OP-2= open form two of ochratoxin;

P= product;

PB= phenobarbital

t½= half-life;

UDS= unscheduled DNA synthesis;

xg= times gravity.

PHARMACOKINETIC EQUATIONS AND THEIR SYMBOLS

$Cp = Ae^{-\alpha t} + Be^{-\beta t}$

Cp= the blood concentration at time t;

A and B are y intercepts;

α and ß are the distribution and the terminal elimination rate constants;

t is time;

$AUC_{0-\infty} = A/\alpha + B/\beta$

$AUC_{0-\infty}$ is the area under the curve from the time zero to infinity;

$Vc = dose/(A+B) \times B.W.$

$Vp = (Vc \times k_{12})/k_{21}$

Vc is the volume of distribution of the central compartment;

Vp is the apparent volume of the peripheral compartment;

B.W. is body weight of the rat;

$k_{21} = (A\beta+B\alpha)/(A+B)$

$k_{12} = \alpha + \beta - k_{21} - k_{10}$

$k_{10} = \alpha\beta/k_{21}$

k_{10} is the overall elimination rate constant;

k_{21} and k_{12} are distribution rate constants;

$Cl_R = Du^{\infty}/ AUC_{0-\infty} = Du^{t*}/AUC_{t1-t2}$

Du^{t^*} is amount of unchanged toxin excreted in the urine over the collection period, $t^* = t_1 - t_2$;

AUC_{t1-t2} is the area under the toxin blood concentration-time curve over the collection period;

t1 is initial time of collection;

t2 is final time of collection;

$Cl_B = D_B^{t^*}/AUC_{t1-t2}$

Cl_B is biliary clearance;

$D_B^{t^*}$ is the amount of unchanged toxin excreted in the bile over the collection period;

$Cl_T = k_{10} \times Vc$

Cl_T is the total clearance, k_{10} and Vc are as defined previously;

$Cl_M = Cl_T - Cl_R - Cl_B$.

Cl_M is metabolic clearance of the administered toxin;

GENERAL INTRODUCTION

Ochratoxin A (OA) is a mycotoxin produced mainly by two toxigenic species of fungi, *Aspergillus ochraceus* Wilhem and *Penicillium verrucosum* Dierckx (de Scott, 1965; Chu, 1974; Frisvad and Samson, 1991). The ochratoxins are a family of structurally related derivatives including ochratoxin A (OA), B (OB), C (OC), 4-hydroxyochratoxin A (OA-OH), ochratoxin α (Oα), ochratoxin β (Oβ), and the open form of ochratoxin A (OP-OA) (van der Merwe et al., 1965a,b; Steyn and Holzapfel, 1967a; Steyn, 1971; Hutchison and Steyn, 1971; Chu et al., 1972; Applegate, 1973; Cole and Cox, 1981; Xiao et al., 1996a). These naturally occurring mycotoxins are found worldwide in food and feedstuffs including various crops and animal tissues and even in human blood (Hult et al., 1979, 1980, 1982 and 1984; Abramson et al., 1983; Steyn, 1984; Krogh et al., 1974b; Williams, 1985; Frohlich et al., 1991; Ominski et al., 1996). OA, OC and the less toxic dechloro analog of OA, OB, are produced by *Penicillium* and *Aspergillus* species of fungi (de Scott, 1965). OA and OC have a similar structure and also similar toxicities. They are extremely toxic to animals, such as pigs, poultry (Chang et al., 1979; Dwivedi and Burns, 1986a,b), and dairy cattle (Still, 1973). The metabolites, OA-OH and Oα, have been found in urine and feces (Chang and Chu, 1977; Hult et al., 1976; Kiessling et al., 1984). OB which has a H-group instead of a Cl-group at C-5, is approximately 10-fold less toxic than OA, while the metabolites of OA, Oα and OA-OH, are either not toxic or are only slightly toxic (Marquardt and Frohlich,

1992). The new discovered form of OA, OP-OA, has been shown to be highly toxic when injected intravenously in rats (Xiao et al., 1996a).

OA is of greatest concern in humans due to its implicated role in an irreversible and fatal kidney disease referred to as Balkan endemic nephropathy and its potent carcinogenic effect (Krogh, 1979; Marquardt and Frohlich, 1992). It is primarily known as a nephrotoxin, a hepatoxin, a carcinogen and an immunosuppressive agent (Dwivedi and Burns, 1984, 1986a, b; Krogh, 1977, 1992; Petkova-Bocharova et al., 1988; Shlosberg et al., 1997). In humans, OA has been detected in both the blood and mothers milk. In Manitoba up to 30% of humans had low but detectable amounts of OA (Frohlich et al., 1991).

The pharmacokinetic profile of OA follows a two compartment model with variations among different animals (Chang and Chu, 1977; Suzuki et al., 1977; Galtier et al., 1979, 1981; Hagelberg et al., 1989). Ochratoxin A is absorbed throughout the gastrointestinal tract and is subjected to intestinal secretion and reabsorption via enterohepatic recycling in the bile and kidney. It is primarily excreted in the urine as Oα and to lesser degree as OA. Small amounts of these compounds are excreted in the feces (Marquardt and Frohlich, 1992). Although some pharmacokinetic studies have been carried out on OA in several different species of animals, there are a limited number of studies on the half-life of OB, and no reports its metabolites including OC, OA-OH, Oα and OP-OA. No studies have been reported on the clearance of any of the ochratoxins from the body. Also, there is limited information available on the nature of metabolites that are formed from OA in the bile and

urine. In addition, most HPLC procedures required that the sample be subjected to a cleanup procedure prior to being analyzed. This is a time consuming and expensive procedure and often results in loss of the more water-soluble metabolites. It would therefore be highly desirable to develop a procedure that does not require a cleanup step. The overall objectives of the research presented in this thesis were: 1) to demonstrate that many metabolites are formed *in vivo* from the different ochratoxins; 2) to develop new procedures to confirm the presence of the ochratoxins in biological samples including a simplified esterification method and a procedure for the interconversion of the ochratoxins into their opened or closed forms; 3) to establish the half-lives of OA, OB, OC, OA-OH, Oα and OP-OA in rats; 4) to obtain information on the clearance of OA and its metabolites in rats via the kidney and the bile, and through metabolism following single intravenous administrations of the different ochratoxins; and 5) to confirm that one of the new metabolites which was discovered in the bile and urine of rats was OP-OA. These studies should therefore provide new evidence on the analysis, confirmation and disposition of ochratoxins in mammals.

LITERATURE REVIEW

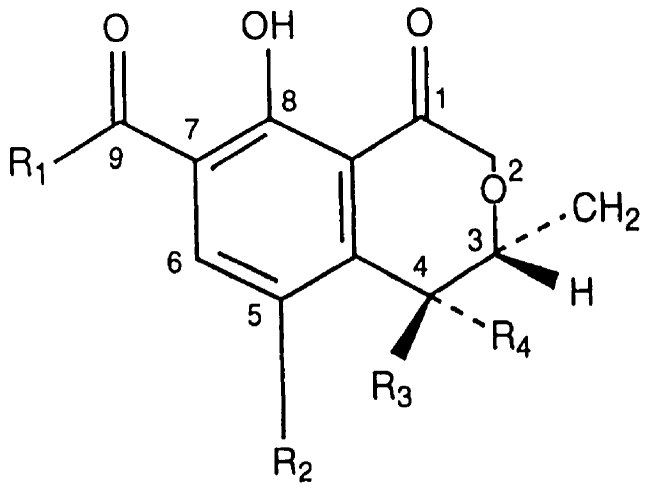
1. Metabolites and Analogs of Ochratoxin A

Ochratoxins are a family of mycotoxins which have structurally related derivatives. Included are ochratoxin A (OA), its analogs including ochratoxin B (OB, OA without chlorine atom) and ochratoxin C (OC, OA ethyl ester), its metabolites including 4-hydroxyochratoxin A (OA-OH), ochratoxin α (Oα, OA without phenylalanine)(van der Merwe et al., 1965a, b; Hutchinson and Steyn, 1971; Steyn and Holzapfel, 1971a; Chu et al., 1972; Steyn, 1984; Hadidane et al. 1992) and the open form of OA (OP-OA) (Xiao et al., 1996a).

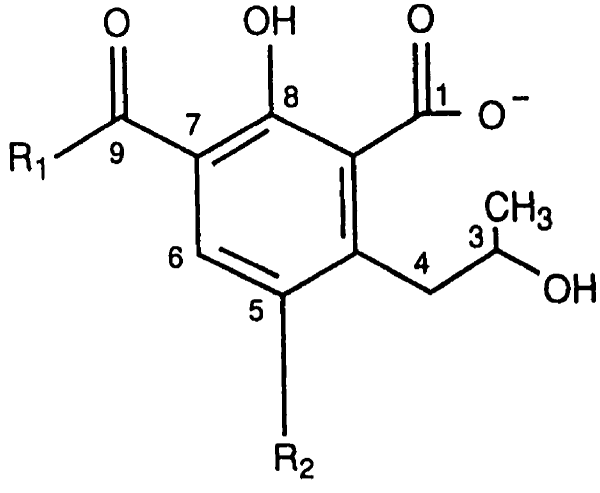
1.1 Ochratoxin A (OA):

Ochratoxin A (Figure 1 and Table 1) was first isolated from a culture of *Aspergillus ochraceus* by van der Merwe et al in 1965. It is a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin-7-L-β-phenylalanine having a molecular weight of 403 D and a molecular formula of $C_{20}H_{18}NO_6Cl$ (Cole and Cox, 1981). Ochratoxin A is a colorless, heat stable and crystalline compound. It is highly soluble in its hydrophobic form (non-charged form), in nonpolar organic solvents such as chloroform and benzene, and in hydrophilic form in polar organic solvents such as methanol and acetonitrile (Roschenthaler et al., 1984). It contains ionizable carboxyl and phenolic hydroxyl groups with the apparent pKa value of its phenolic group being 7.0 (Chu et al., 1972; Xiao et al., 1996a). It is highly

Fig 1. I, Structure of ochratoxin A (OA) and its analogs; II, Structure of open ochratoxin A (OP-OA). See Table 1 for identification of the R groups according to Marquardt and Frohlich (1992).



I



II

TABLE 1

The Structure of the Different *R* Groups of Ochratoxin A

Fig. No.	Common name	Abbreviation	R_1	R_2	R_3	R_4	R_5
I	Ochratoxin A	OA	Phenylalanyl	Cl	H	H	H
I	Ochratoxin B	OB	Phenylalanyl	H	H	H	H
I	Ochratoxin C	OC	Phenylalanyl, ethyl ester	Cl	H	H	H
I	Ochratoxin α	Oα	OH	Cl	H	H	H
I	Ochratoxin β	Oβ	OH	H	H	H	H
I	4*R*-Hydroxyochratoxin A	OA-OH	Phenylalanyl	Cl	H	OH	H
I	4*S*-Hydroxyochratoxin A	OA-OH	Phenylalanyl	Cl	OH	H	H
I	10-Hydroxyochratoxin A	OA-OH	Phenylalanyl	Cl	H	H	OH
II	Open ochratoxin A	OP-OA	Phenylalanyl	Cl	—	—	—

soluble in aqueous solutions, such as 0.1 M $NaHCO_3$ a pH value greater than its pKa value. Ochratoxin A in absolute ethonal has ultraviolet absorbance maxima at 213 (ϵ 36,800) and 332 nm (ϵ 6,400). In aqueous solutions OA has absorbance maxima at 333 and 380 nm at pH 1.5 and 8.5, respectively (Roschenthaler et al., 1984). Ochratoxin A is known to bind tightly with serum protein particularly albumins (Chu, 1971). Chu et al. (1972) proposed that the dissociation of the phenolic hydroxyl groups of OA was required for its toxicity. Hasinoff et al. (1990) reported that the iron-complex of OA produced highly toxic hydroxyl radicals in the presence of NADPH and a cytochrome P-450 reductase system. These radicals, therefore, may be partly responsible for its toxicity (Marquardt and Frohlich, 1992). Xiao et al. (1996a) has suggested that the toxicities of OA were associated with its isocoumarin moiety but that neither the dissociation of the phenolic hydroxyl group nor the iron-chelating properties of OA were directly related to its toxicities. Detailed studies on the role of iron and OA on the production of free-radicals in a model system have been presented by Hoehler et al. (1996a). Among the ochratoxins, OA is the most toxic to animals (Cole and Cox, 1981). It can be hydrolyzed by inorganic acid and by enzymes such as carboxypeptidase A into phenylalanine and carboxylisocoumarin (ochratoxin α, Oα)(Fig 1 and Table 1), a form that is not toxic to animals (Steyn, 1984).

1.2 Ochratoxin B (OB):

Ochratoxin B (Figure 1 and Table 1) which is the dechlororinated form of OA (369 MW), is considerably less toxic than OA (Cole and Cox, 1981). It has a pKa of 7.8 to 8.0

(Chu et al., 1972; Xiao et al., 1996a). OB can be hydrolyzed into its isocoumarin moiety (Oβ) by bovine carboxypeptidase or by various rat tissue extracts at a much faster rate than OA (Doster and Sinnhuber, 1972b). The toxicity of OB to animals is approximately 10-fold lower than that of OA (Chu, 1974).

1.3 Ochratoxin C (OC):

Ochratoxin C (Figure 1 and Table 1) is simply the ethyl ester of OA and has a pKa of approximately 7.1 to 7.2 (Chu et al., 1972; Xiao et al., 1996a). OC can be rapidly converted into OA *in vivo* (Fuchs et al, 1984). It therefore has a similar toxicity and pattern of metabolism to that of OA (Marquardt and Frohlich, 1992).

1.4 Ochratoxin α (Oα):

Ochratoxin α (Figure 1 and Table 1) is a non-toxic metabolite of OA which is primarily excreted in the urine and feces of animals fed OA contaminated diets. It has a low molecular weight (256 MW), a pKa of 11.0 to 11.6 (Xiao et al., 1996a) and is water soluble (Chu et al., 1972, Xiao et al., 1996a).

1.5 Hydroxyochratoxin A (OA-OH):

Hydroxyochratoxin A (Figure 1 and Table 1) occurs in at least three isomeric forms, 4S-, 4R- and 10-hydroxy OA. OA-OH is produced in both a culture of *A. ochraceus* and in the urine of animals (Hutchinson and Steyn, 1971; Størmer and Pedersen, 1980; Størmer et al., 1981; Cole and Cox, 1981; Xiao et al., 1996b). The 4-hydroxyochratoxins also are relatively non-toxic to animals and therefore can be considered to be detoxified forms of OA

(Cole and Cox, 1981).

1.6 Lactone opened form of OA (OP-OA):

The lactone opened form of OA is a newly discovered form of OA. It is formed by the hydrolysis of the lactone of OA at a high pH or *in vivo* by an unknown metabolic pathway (Fig1 and Table 1) (Xiao et al., 1995a). The opened lactone ring can be closed by acidification with 6 N HCl at 25°C (pH < 1.0) for 6 h resulting in the reformation of OA. OP-OA was first discovered in bile of rat and has a similar toxicity to that of OA when administered intravenously to the rats (Xiao et al., 1996a).

2. Isolation, Analysis and Confirmation of Ochratoxins

Ochratoxin A was first isolated from moistened corn meal and other agricultural commodities after colonization by *A. ochraceus* (van der Merwe et al., 1965a, b). The original method of purification involved the initial extraction of ochratoxin A and B from molded corn meal with chloroform-methanol and partitioning into aqueous sodium hydrogen carbonate. The ochratoxins were obtained from the aqueous layer by acidification and extraction into chloroform followed by purification using column chromatography. Several other modifications of this procedure have been utilized for the isolation OA, OB and their metabolites (van der Merwe et al., 1965 b). Peterson and Ciegler (1978) reported the purification of ochratoxin A by using liquid-liquid chromatography (HPLC). The procedure is presented in Fig 2. These have been reviewed in detail (Chu, 1974, 1992;

Fig 2. General procedure for purification of ochratoxin A (OA) from fermented wheat according to Peterson and Ciegler (1978).

1 Fermented wheat (2.274 g)

↓

2 Ground in Waring blender with equal weight of water

↓

3 Extracted twice with methanol

↓

4 Methanol solution (18 500 ml, 6.13 g of OTA)
Sodium chloride (3 M) added
Extracted twice with methylene chloride

↓

5 Methylene chloride solution (3760 ml, 3.76 g of OTA)
Extracted with 0.2 M sodium bicarbonate

↓

6 Bicarbonate solution
Acidified to pH 2.5 with concentrated HCl
Extracted with methylene chloride

↓

7 Methylene chloride solution (1750 ml, 3.5 g of OTA)
Filtered through sodium sulphate
Evaporated to dryness at room temperature

↓

8 Ethyl acetate solution
Filtered and evaporated to dryness

↓

9 Ethyl acetate solution (32 ml, 3.2 g of OTA)
Injected into high-pressure liquid chromatograph

↓

10 Active fractions evaporated to dryness

↓

11 OTA crystallized from benzene (2.7 g of OTA)

Nesheim, 1976).

Several procedures have been used to extract ochratoxin from tissues for analysis. Some of the factors that must be considered have been reviewed by Chu et al. (1972). One of the most commonly used procedures for biological samples is that developed by Hult et al. (1979) which involves the homogenization of tissues in an acidified $MgCl_2$ solution, extraction of OA into chloroform, drying the extract and reconstituting it in a solvent such as methanol. Nesheim (1969, 1973) developed a method for the extraction and cleanup of esters of ochratoxin A and B. This involved their extraction with water-chloroform (1+9) followed by chromatography of the extract in aqueous sodium bicarbonate on a diatomaceous earth column. The OA and OB esters were removed from the column with hexane-chloroform (1+99). Several other methods have been reported in the literature (Betina, 1989). An initial cleanup procedure is required in all reported methods. Silica-, cyano- or Sep-Pak C-18-cartrides, or thin-layer chromatography (TLC) have been used (Frohlich et al., 1988). Recently Xiao et al. (1996b) used a gel filtration column (Sephadex G25) to remove small contaminates.

The most widely used method for quantitating OA and its metabolites has been using a HPLC reversed-phase column (Xiao et al., 1996a,b). Immunoassays have also been widely used for regular purposes (Chu, 1992). A modified method for the analysis of tissues, including liver and kidney samples, has been reported by Clarke et al. (1994). Nesheim (1969) developed a method for quantitating OA and OB on TLC plates by measuring their

fluorescence intensity. Recent modifications of the HPLC procedure has involved the elution of the ochratoxins from a reversed-phase column using a gradient rather than an isocratic mobile phase (Ominski et al., 1996; Xiao et al., 1996a,b). The advantage of this procedure is that the gradient can be adjusted so that contaminates do not co-elute with the particular form of ochratoxin that is being detected, each of the metabolites of OA can be separated from one another and the peaks are sharp and well defined. Under such conditions a prior cleanup of the sample is not required as the fluorescent contaminate can be flushed from the system prior to elution of the desired compounds or can be eluted at a time that is different to that of the compound being detected. The limit of detection was about 0.5 ng/g (Scott et al., 1991).

There is also a need to develop simple methods to confirm the presence of OA and its metabolites in tissue, especially in samples that are analyzed using 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). Other procedures involve NMR analysis on purified samples, the use of independent analytical methods such as immunoassays, and the conversion of OA into modified forms such as it methyl ester (Nesheim, 1976) or hydrolysis into its alpha form (Doster et al., 1972; Madhyastha et al., 1992a).

3. Occurrence of Ochratoxin A:

3.1 Ochratoxin-producing fungi:

Fungi (molds) are one of the most ubiquitous types of microbes in nature. Important filamentous fungal genera invading cereal grains in storage are a large number of species of *Aspergillus* and *Penicillium* (Krogh, 1987), several that produce OA (Frisvad and Samson, 1991). *Aspergillus ochraceus* Wilhelm was the first fungus found to be an ochratoxin producer (van der Merwe et al, 1965a, b). *A. ochraceus* has been previously and incorrectly referred to as *A. alutaceus* (Chelack et al., 1991a,b). Throughout this thesis this species will be referred to as *A. ochraceus*. *Penicillium verrucosum* Dirckx (Frisvad and Samson, 1991), types 1 and 2, is the main *Penicillum* producer of ochratoxin.

3.2 Environmental factors

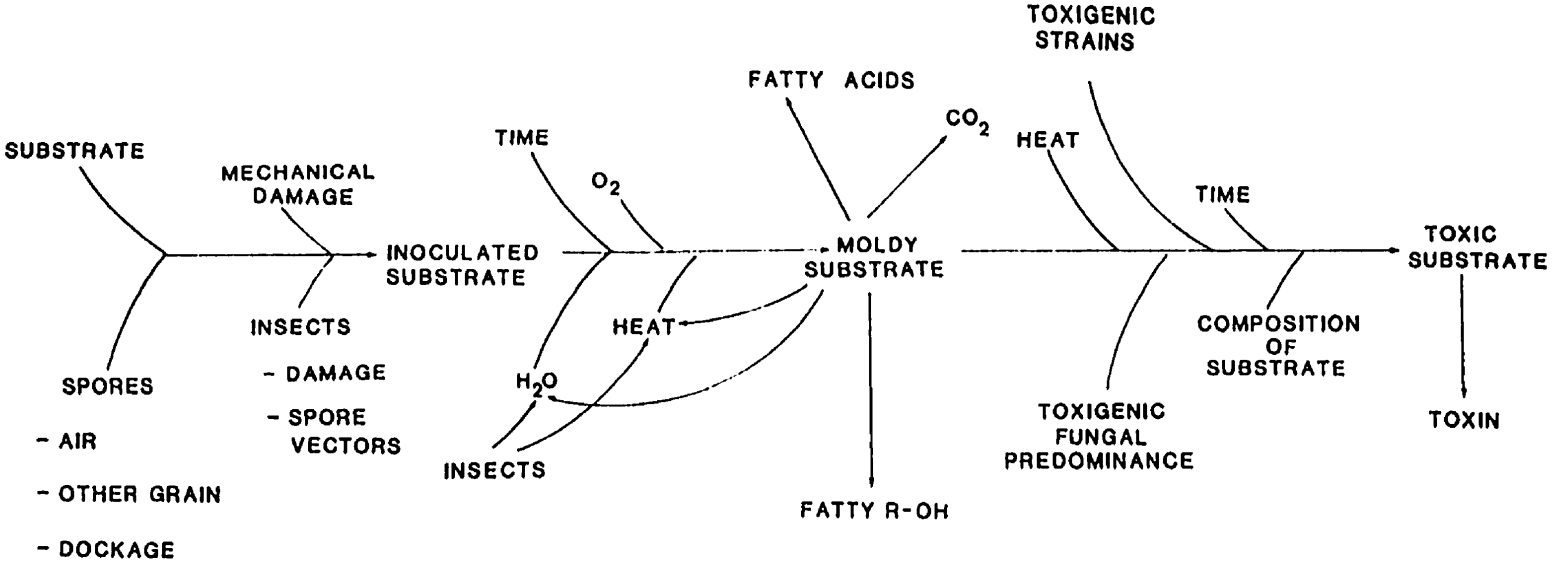
The amount of OA produced by any species of fungi is influenced by many factors, including water activity (a_w), temperature, type of substrate, presence of competitive microflora, strain of fungi, and integrity of the seed. The minimum a_w values for OA production by *A. ochraceus* and *P. verrucosum* are in the range from 0.83 to 0.90. The optimum a_w values at 24°C are from 0.95 to 0.99 (Northolt et al., 1979). The temperature range for production of OA by *A. ochraceus* is from 12 to 37°C at the optimum a_w which occurs in warm climatic zones, such as Yugoslavia and Australia. In contrast, that for *P. verrucosum* is from 4 to 31°C which occurs in the colder climatic zones, such as Scandinavia and Canada (Krogh, 1987). The growth of the storage molds is greatly influenced by water

content of the substrate. In general, if the moisture content of stored grains increases above 14-16%, mycotoxin formation may occur and at moisture levels of 20-25% maximum production may be achieved. Abramson et al. (1980) reported that OA formed in barley and wheat but not oats at 21% moisture content and at 12-29% in stored grain. Frohlich et al. (1991) obtained grain from farm bins in different areas in Manitoba and incubated these samples in the presence of 24 to 25% moisture at 28 °C for 14 days. This resulted in considerable mold infestation in all samples from indigenous spores present on the grain with 35% of the sample containing OA, half of which were at concentration above 0.5 ng/kg. Fungal growth, crop spoilage and mycotoxin formation result for the interaction of numerous factors illustrated in Figure 3 (Abramson, 1991).

3.3 Natural occurrence:

Aspergillus and *Penicillium* species are widespread in nature. *A. ochraceus* have been isolated from a variety of agricultural commodities including stored feed wheat, oats and barley (Abramson et al., 1983), a variety of foods, feedstuffs and stored grains (Wallace and Sinha, 1962; Shotwell et al., 1969; Yamazaki et al, 1970; Doupnik and Peckham, 1970; Pavlovic et al., 1979), cereals and sorghum (Scott, 1965), black and red peppers (Christensen et al., 1967; Christensen and Kaufman, 1969), hay (Still, 1973), corn (Richard et al., 1969), poultry feed (Bacon, 1973), rice (Udagawa et al., 1970., Yamazaki et al., 1970), dried peas and beans (Williams, 1985; Fuchs et al., 1991), coffee beans (Levi et al, 1974), wheat (Manning and Wyatt, 1984), bread and flour (Osborne, 1980, Viscontin and

Fig 3. Some factors affecting mycotoxin formation in stored cereal crops according to Abramson (1991).


SUBSTRATE
MECHANICAL DAMAGE
INOCULATED SUBSTRATE
SPORES
- AIR
- OTHER GRAIN
- DOCKAGE
INSECTS
- DAMAGE
- SPORE VECTORS
TIME
O_2
H_2O
INSECTS
HEAT
FATTY ACIDS
CO_2
MOLDY SUBSTRATE
FATTY R-OH
TOXIGENIC STRAINS
HEAT
TIME
COMPOSITION OF SUBSTRATE
TOXIGENIC FUNGAL PREDOMINANCE
TOXIC SUBSTRATE
TOXIN

Betulaceae, 1983), and country cured ham (Escher et al., 1973). In Canada and United States the percentage of feed and food that were contaminated ranged from 1 to 14.2% with its concentration being from 5 to 27,500 μg/kg (ppb) (Betina, 1984). Recently, Abramson (1997) reported OA contamination up to 1,600 μg/kg in mixed grains collected in Western Canada during the period from 1982-1994.

3.4 Residues of OA in animal blood and tissues:

Ochratoxin A has been detected in the blood of swine in several countries including Canada (Marquardt et al., 1988; Frohlich et al., 1991; Ominski et al., 1996), Germany (Unglaub and Holl, 1990), Sweden (Hult et al., 1984) and Japan (Ueno et al., 1991), and in Denmark in swine tissues (Hald, 1991). In Manitoba, 36% of the serum samples collected from 1,600 pigs contained detectable levels of OA (Ominski et al., 1996). In July, 65% of the samples contained OA, compared with 38, 21 and 17% in April, October and January, respectively. Furthermore 24% of the samples collected in July contained greater than 15 ng/ml OA, while only 2, 9 and 1% of the samples collected in April, October and January, respectively, contained greater than 15 ng/ml OA. The finding that a high proportion of samples collected in May, June and July contained higher levels of OA than in the winter months, suggests that the risk of fungal growth in stored grains is greater in the spring and early summer owing to warmer temperatures and possibly higher moister levels than in winter, which may lead to increase fungi growth and corresponding OA production (Marquardt et al., 1988). In addition, residues of OA have been detected in the kidneys

(77μg/kg), liver (0-21μg/kg), and blood (36-77μg/kg) of swine from nephropathic areas in Yugoslavia (Pepeljnjak and Cvetnic., 1985). Spontaneous occurrence of OA in the kidneys of fattening pigs was noted in Sweden (Rutqvist et al., 1977, 1978) and in porcine kidney, and serum samples in Poland (Golinski et al., 1984, 1985). Krogh et al. (1976) reported the occurrence of OA residues in tissues from slaughtered pigs with nephropathy and reported a correlation between OA concentration and swine nephropathy. Feeding experiments with bacon pigs ingesting OA contaminated barley revealed residues of OA in the kidney, liver, lean and fat (Madsen et al., 1982). Residues of OA were also found in the blood and tissues of the poultry when OA contaminated feed was consumed (Dwivedi and Burns, 1986a,b). Included are broiler chickens and laying hens dosed over a long period of time with low concentrations of OA (Micco et al., 1987). It has been suggested that the concentration of OA in the blood of animals can be used to estimate the concentration of the toxin in the feed (Hult et al., 1979, 1980).

The natural occurrence of OA residues has been reported in human blood (Hult et al, 1982; Petkova-Bocharova, 1988; Frohlich et al., 1991; Breitholtz-Emanuelsson et al, 1994; Radić et al., 1997; Scott, 1998) and mothers' milk (Breitholtz-Emanuelsson, 1993). In Manitoba up to 30% of the population in one survey, had low but detectable amounts of OA (Frohlich et al., 1991). Recently Scott et al. (1998) reported that OA was detected in the human blood in Canada, with these concentrations being particularly high in Winnipeg (1.73 μg/ml). In general, the concentration and frequency of contamination was higher in feeds

than that in foods. Therefore, OA appears to be diluted at the higher end of the food chain but, nevertheless, may result in serious health effects and economic losses because of its high toxicity. These observations are supported by the implication that OA is a causal agent of a serious kidney disease in the Balkan countries (Balkan endemic nephropathy)(Krogh, 1978, 1979; Hult et al., 1982) and/or renal tumors associated with this disease (Castegnaro et al., 1987).

4. Ochratoxicosis in Animals and General Toxicity of Ochratoxin A.

4.1 Ochratoxicosis:

Ochratoxin A, a nephrotoxic mycotoxin, affects the kidneys of animals naturally exposed to this mycotoxin (Krogh, 1977). Changes in the renal function of pigs exposed to OA include degeneration of the proximal tubules, intestinal fibrosis and hyalinization of the glomeruli. As a result, there is a decrease in concentration of the urine and an increase in glucose excretion (Krogh et al., 1974a,b; Krogh, 1978, 1987). Extrarenal effects often occur in animals exposed to levels of OA in feed greater than 5 to 10 ppm. These effects included enteritis, necrosis of lymphoid tissue, and fatty changes in the liver (Szczech et al., 1973).

The first documented field outbreak of ochratoxicosis in poultry resulted in a large increase in mortality in a house containing approximately 16,000 young turkeys. A total of five independent episodes of ochratoxicosis in about 970,000 turkeys, two episodes in 70,000 laying hens, and two episodes in 12 million broiler chickens in the United States

were investigated by Hamilton et al. (1982). Toxin levels in the diet ranged from 0.3 to 16 mg/kg of diet. These infestations caused reductions in efficiency of feed utilization and increases in mortalities ranging from 21 to 58%. Marquardt and Frohlich (1992) reported that the main clinical patterns associated with acute ochratoxicosis include initial anorexia, weight loss, emesis, and polydipsia, polyuria, passage of clots of blood-stained mucus from the rectum, dehydration, prostration, and death within two weeks after administration of toxin. The symptoms manifest themselves earlier when a higher dose of OA is fed (Chu, 1974).

In poultry there are reductions in serum levels of total protein, albumin, globulin, urea nitrogen, cholesterol, triglycerides, and potassium and increases in uric acid and creatine levels and in the activities of serum phosphatase, γ-glutamyltransferase, and cholinesterase (Huff et al., 1988a; Sreemannaryana et al., 1989). Traits such as kidney weight and the serum protein concentrations and metabolites and the activity of certain enzymes can be used as sensitive and earlier indicators of ochratoxicosis (Huff et al., 1988b). It has been reported that a decrease in renal phosphoenolpyruvate carboxykinase (PEPCK) activity is a highly sensitive and specific indicator of OA toxicity in pigs, but not in rats (Krogh et al., 1988). The activity of renal PEPCK and γ-glutamyltransferase decreased by 40% in pigs within one week of being fed 2,000 or 1,000 mg of OA/kg of diet (Krogh et al., 1988).

Krogh et al. (1976) reported that pigs fed diets containing 200 to 4,000 μg/kg of OA developed nephropathy after 4 months at all levels of exposure. All the lesions in the pigs

were confined to the kidney, and most of the renal disturbances were associated with damage to the proximal tubules. Changes in tubular function were indicated by decreased transport maximum of p-aminohippuric acid (Tm-PAH), a decreased Tm-PAH/inulin clearance, an increased excretion of glucose, and a reduced ability to produce a concentrated urine. There was a dose-response-related increase in protein excretion in the urine consisting mainly of albumin and a "glomerular" proteinuria. Polydipsia and significant increases in the concentration of creatinine in the blood and urine occurred in the highest exposure group after 4 to 6 wk. These functional changes in renal structure were observed at all exposure levels (Marquardt and Frohlich, 1992).

The target organ of OA for turkeys, as reported for other species, seems to be the kidney, although liver, gastrointestinal tract, lymphoid organs, skeletal system, hematopoietic tissues, and the reproductive organs can be affected (Burns and Dwivedi, 1986).

Ochratoxicosis has rarely been reported in ruminants, presumably because of the ability of ruminal microorganism to hydrolyze OA to Oα, a nontoxic form of OA (Sreemannarayana et al., 1988; Kuiper-Goodman and Scott, 1989; Xiao et al., 1991a, b).

4.2 General toxicity.

The acute toxicity of OA has been reported for different species including chickens, ducklings, turkeys, rats, sheep, swine and rainbow trout (Peckham et al., 1971; Chu, 1974; Dwivedi and Burns, 1984, 1986a,b; Kuiper-Goodman and Scott, 1989; Marquardt et al.,

1990). The LD_{50} (median lethal dose) of OA given orally to one-week-old New Hampshire Leghorn cross chicks was 166 μg/kg (Chu et al., 1972). In broiler chicks the LD_{50} for day-old chicks was 2.14 mg/kg body weight, and for 3 week-old birds was 3.6 mg/kg body weight (Peckham et al., 1971). Initial studies on day-old ducklings indicated that the oral LD_{50} of OA was 25 μg/kg (Van der Merwe et al., 1965a). Chang et al. (1981) found the oral LD_{50} of OA for day-old and 3 week-old turkey poultry to be 4.63 and 7.84 mg/kg body weight, respectively, while the corresponding intraperitoneal values were 0.16 and 0.34 mg/kg body weight, respectively. The LD_{50} of OA for beagle dogs up to 14 days of age was estimated to be 0.2-0.3 mg/kg daily within 14 days (Chu, 1974). Studies on the rat indicated that the LD_{50} of OA when administered by oral-stomach tube was 20-22 mg/kg body weight. The oral LD_{50} in 24 h old infant rats was 3.9 mg/kg (Hayes et al., 1977). The 24 h LD_{50} of OA in sheep infused intravenously with OA was estimated to be 1 mg/kg (Munro et al., 1973). The LD_{50} in swine was found to be 1-2 mg/kg when the toxin was fed daily over a 5-6 day period (Chu, 1974). The LD_{50} value of OA to 60 day old rainbow trout following intraperitoneal injections of the toxin was 4.65 mg/kg body weight (Doster et al., 1972). The acute intraperitoneal toxicity of OA over a seven day period for adult female Swiss mice was found to be 48 mg/kg and to be 10 mg/kg when daily dosed for 10 days (Prior and Sisodia, 1982).

4.3 Other toxicity:

Ochratoxin A damages the kidneys of a wide variety of domestic and wild animals

that consume contaminated feed (Krogh, et al. 1979). High concentrations of dietary OA also can cause liver damage as well as intestinal necrosis and hemorrhage. Lymphocyte infiltration occurred in the liver along with lymphocytolysis in lymphoid organs. Necrotic changes in periportial cells were observed in rats given the LD_{50} (20 mg/kg body weight) dose of OA (Terao and Ueno, 1978). The effects of OA on liver are apparently much less pronounced and specific than those of aflatoxins (CAST, 1989).

Ochratoxin A has been shown to be teratogenic in at least one mammalian species (Hayes, 1981), immunosuppressive and carcinogenic (CAST, 1989). Intraperitoneal administration of OA to pregnant mice had a teratogenic effect on surviving pigs, increasing fetal death and decreasing fetal weight (Hayes et al., 1974). Immunosuppression by OA has been studied mostly in poultry, with evidence suggesting that this mycotoxin has an effect on immunoglobulins and phagocytic cells. Cellular immune responses in poultry were affected by OA more in broilers than in quail or turkeys (Burns and Dwivedi, 1986). Tumorigenesis/carcinogenesis has been reported in laboratory animals (Ueno, 1984). Renal and hepatic neoplasmas have been induced in laboratory mice fed 40 ppm OA for 20 months (Bendele et al., 1985; Ueno, 1984). OA is not considered to be mutagenic (for reviews, see Kuiper-Goodman and Scott, 1989; Dirheimer and Creppy, 1991; IARC, 1993; Pfohl-Leszkowicz, 1994).

Ochratoxin A also has an effect on glucose and insulin metabolism (Subramanian et al., 1989), promotes glycogen accumulation in liver (Pitout, 1968; Huff et al., 1979) by

inhibiting the activation of glycogenolysis via the cAMP-dependent protein kinase (Warren and Hamilton, 1980a), and as indicated previously inhibits the activity of a key gluconeogenic enzyme in the kidney, PEPCK (Meisner and Meisner, 1981; Meisner and Krogh, 1986; Krogh et al., 1988). The concentration of OA required to inhibit 50% of PEPCK in pigs is 4 μg/kg BW, whereas in rats, 1,000 to 2,000 μg/kg body weight is required (Marquardt and Frohlich, 1992). One of the unique properties of OA is its capability for non-covalent interaction with serum albumin and other proteins but not with DNA and RNA (Chu, 1974; Kiessling, 1986).

4.4 Mechanism of ochratoxins.

Several hypotheses have been put forward concerning the toxicomechanism of ochratoxins. The hypothesis of Creppy et al. (1983a,b) seems to be the most prominent one. They suggested that competitive inhibition of aminoacyclation of $tRNA^{phe}$ by OA is the primary cause of OA toxicity. The inhibition of tRNA-synthetase is accompanied by reduced protein synthesis in many different microorganisms and hepatoma cells (Röschenthaler et al., 1984; Meisner and Krogh, 1986). Further studies suggested that OA might affect the turnover of macromolecules rather than their synthesis and that this effect might be attributed to some primary mechanisms (Marqardt and Frohlich, 1992). Creppy et al. (1986) studied the effect of OA on macromolecule synthesis *in vitro* in kidney cells and suggested that the phenylalanine moiety of OA was involved in the toxic action of OA. Analogs of OA in which the phenylalanine moiety had been replaced by other amino acids inhibited, in

similar manner, their corresponding aminoacycl-tRNA synthetases and as a consequence protein synthesis (Creppy et al., 1983a,b). Creppy et al. (1990) reported that tyrosine-ochratoxin A was formed (up to 6% of total mycotoxin added) when a tissue preparation was incubated with labeled ^{3}H-OA and unlabeled phenylalanine, indicating that ochratoxin can act as a substrate for phenylalanine hydroxylase. *In vivo* tryosine-ochratoxin A is also found in liver of poisoned animals. Ochratoxin A affects DNA, RNA, and protein synthesis in many different organisms, presumably due to an effect by the phenylalanine moiety of the molecule. Protein and mRNA pools are reduced in kidney cells by 30 to 40%, whereas renal gluconeogenesis and cytosolic PEPCK activity can be almost completely inhibited in rats and swine by OA (Meisner and Meisner, 1981; Meisner and Krogh, 1986; Krogh, 1987). Dörrenhaus and Follmann (1997) reported that OA induced unscheduled DNA synthesis (UDS) in a concentration-dependent manner to inhibit the proliferation of the epithelial cells in cultures from the porcine urinary bladder. They also indicated that in cultured rat hepatocytes induction of UDS is relatively weak whereas in urothelial cells this effect was significant. Ochratoxin α and OB have no effect on the phenylalanyl-tRNA synthetase (Roth et al., 1989). The effect of OA on protein synthesis is followed by an inhibition of RNA synthesis, which might affect proteins with a high turnover (Dirheimer, 1996). Analogs of OA in which the pheylalanine moiety had been replaced by other amino acids inhibited in a similar manner (Creppy et al. 1983a).

The second major biochemical effect of OA described is the inhibition of

mitochondrial respiration correlated with a depletion of ATP as a consequence of an inhibition of intramitochondrial phosphate transport (Moore and Truelove, 1970; Wei et al., 1985). During in vitro studies of the rat liver mitochondria it was observed that OA inhibited the respiration of whole mitochondrial membrane (Meisner and Chan, 1974). Ochratoxin A has also been shown to alter mitochondrial morphology after *in vivo* administration (Suzuki et al., 1975).

The third mechanism related primarily to liver toxicity of OA is its effect on hepatic microsomal calcium sequestration which could be observed both *in vitro* and vivo in rats at high dosage (10 mg/kg B. W.)(Khan et al., 1989). Distribution of calcium homeostasis might be attributed to an impairment of the endoplasmic reticulum membrane by enhanced lipid peroxidation caused by OA (Rahimtula et al., 1988; Omar et al., 1991). The ability of OA to induce lipid peroxidation has been linked to genotoxicity caused by DNA adduct formation (Pfohl-Leszkowicz et al., 1993). Ochratoxin A stimulated lipid peroxidation by forming a complex with iron, facilitating the reduction of iron (Omar et al., 1990). Hasinoff et al. (1990) reported that the iron-complex of OA produced the extremely toxic hydroxyl radical in the presence of the NADPH-cytochrome P-450 reductase system. They suggested that this radical species may be partly responsible for OA toxicity. Xiao et al. (1996a) suggested that the toxicity of OA was associated with its isocoumarin moiety but that neither the dissociation of the phenolic hydroxyl group nor the iron chelate was directly related to its toxicity. Hoehler et al. (1996a) carried out studies in a bacterial culture on the generation

of free-radicals by OA using electron paramagnetic resonance (EPR) spectroscopy. They showed an enhanced free radical generation in the presence of OA and most of its analogs. The EPR signals were further enhanced by the addition of Ca^{2+}, a calcium ionophore and an ATPase uncoupler, whereas they were eliminated by incubating the growing cells with vitamin E. The spin adduct hyperfine splitting constants indicate the presence of α-hydroxyl-ethyl radicals, which were generated from hydroxyl radicals and ethanol. The results suggested that OA induced free radical production in their model system by enhancing the permeability of cellular membranes to Ca^{2+}.

Malaveille et al. (1994) demonstrated in *E. coli* that β-lyase action on a cysteiny-OA conjugate is probably associated with the cytotoxic reaction. Kidney toxicity from certain chemicals has been found to result from a β-lyase cleavage of cysteinyl conjugates. This indicates a possible biochemical mechanism for the nephrotoxic action of OA. The genetic control of expression of both the primary activation and conjugation of OA with GSH and the subsequent reactivation by β-lyase could play a role in the toxicity of OA.

4.5 Detoxification and reduction of ochratoxin A-induced toxicity.

Several procedures have been used in an attempt to reduce the toxicity of OA including extraction, heat treatment (Josefsson and Moller, 1980; Bruinink et al., 1997), ammoniation (Chelkwski et al., 1981), and ensiling (Rotter et al., 1990). Nonspecific adsorbants, such as charcoal, have also been tested and shown to be rather ineffective (Rotter et al., 1989a). The antioxidant vitamins (A and C) have been shown to be partially

protective in poultry (Haazele et al., 1993) and rats (Hoehler et al., 1996b) against some of the pro-oxidant effects of OA.

Recently, Baudrimont et al. (1997) proposed that ochratoxin A , a structural analog of phenylalanine in the phenylalanine-tRNA aminoacylation reaction, constituted the main mechanism of OA-induced cytotoxicity. An attempt to prevent its toxic effect, mainly the inhibition of protein synthesis, has been made using aspartame (L-aspartyl-L-phenylalanine methyl ester) a structural analog of both OA and phenylalanine. Aspartamine (A_{19}), at ten-fold higher concentrations than OA (100-1000 μM), was found to be partially protective against OA-induced inhibition of protein synthesis in Vero cells, and to be more effective when added 24 h prior to the toxin (IC_{50} 34 μM) than when they are added together (IC_{50} 22 μM). A_{19} (250 μM) prevented the OA-induced leakage of certain enzymes, including lactate dehydrogenase, γ-glutamyl transferase, alkaline phosphatase into the culture medium, and the concomitant decrease of their intracellular activity in OA (25 μM)-treated cells. In vitro, A19 (10μg/ml) also prevents OA (20-500 μg/ml) binding to plasma proteins. In addition, it does not have any known side effect in humans and in animals. Creppy et al. (1995) concluded that A_{19} is the best candidate for preventing the OA-induced subchronic effects, but no in vivo studies have been carried out with this compound. Studies in vivo with the addition of phenylalanine or protein to the diet of animals, however, have demonstrated that these compounds were relatively ineffective, expensive and, in the case of phenylalanine, can cause an amino acid imbalance (Bailey et al., 1989, 1990; Rotter et

al., 1989b). It may therefore be concluded that although some of these methods have shown promise, most have only a minor effect on the tolerance to or the disposition of OA in animals and that additional research must be carried out with other suggested methods (Marquardt and Frohlich, 1992).

In contrast to the above procedures some treatments offer more promise. The feeding of diets containing OA to ruminants may be an effective means of detoxifying OA as rumen microorganisms are able to hydrolyze OA to non-toxic Oα (Sreemannarayana et al., 1988; Xiao et al., 1991a,b). Moroi et al. (1985) reported a reduction in OA toxicity in mice treated with phenylalanine and phenobabital (PB) for 1 week as the LD_{50} increased to 1.5-2.0 times compared to the untreated controls. The result suggested that an enhanced metabolism of OA into OA-OH caused the decrease in the toxicity of OA in PB-pretreated mice. Størmer et al. (1985) indicated that ochratoxin B considerably reduces the toxic effects of ochratoxin A.

Recent studies with rats have indicated that cholestyramine (CHA), an ion-exchange resin with a high affinity for bile salts (Kos et al., 1991), can decrease the plasma concentration of OA and increase its fecal excretion (Madhyastha et al., 1992b). Kerkadi et al. (1998) showed that CHA decreased the concentration of OA in plasma when animals were fed semisynthetic diets containing 1 or 3 ppm of this toxin. This was associated with an increased excretion of OA in the feces and a decreased excretion in the urine. Enzymuria and renal morphology revealed that dietary CHA can decrease OA-induced nephrotoxicity,

probably by reducing renal exposure to the toxin. These data suggest that the best procedures for reducing OA toxicity appear to be those that increase its rate of metabolism (conversion to Oα or OA-OH) or reduce its uptake (i.e., the feeding of semispecific adsorbents such as cholestyramine). The antioxidant vitamins may protect against the prooxidant effects of OA while phenylalanine or its analogs (aspartamine, A_{19}) would offer protection by competitively excluding the binding of OA to macromolecules.

5. Metabolism of Ochratoxin A.

Studies on the turnover of OA in rats showed that OA was cleaved into dihydroisocoumarin (Oα) and phenylalanine by carboxypeptidases A and α-chymotrypsin which are produced by the pancreas of animals (Pitout, 1969; Nel and Purchase, 1968) and by enzymes produced by intestinal microorganisms (Madhyastha et al., 1992a). There is limited hydrolysis of OA by extracts of various tissues of rats including those from the liver (Doster and Sinnhuber, 1972; Madhyastha et al., 1992a). Oα is considered to be non-toxic to animals (Chu et al, 1972) and is found as a metabolite of OA in the feces and urine of animals which have received OA either intravenously or orally (Patterson et al., 1976, Galtier et al., 1979; Storen et al., 1982; Sreemannarayana et al., 1988; Mahdyastha et al., 1992a). Hydrolysis of OA in the gastrointestinal tract of the ruminant greatly reduces the amount of OA entering the systemic circulation (Sreemannarayana et al., 1988). Studies with ruminants revealed that up to 90% of the administered toxin was converted to Oα in the

rumen which suggested that the microorganisms in the rumen content may play an important role in the detoxification of OA (Xiao et al., 1991a,b). The bioavailability of OA in ruminant cattle was only 26-36% of that in preruminant cattle (Sreemannarayana et al, 1988). Degradation of OA by rumen microorganisms has been shown to be very efficient as 40% of OA incubated with rumen fluid is converted to Oα within 1-4 h (Hult et al., 1976). The rate of hydrolysis of OA by the rumen is dependent on the species of the microflora that are present in the rumen which in turn are affected by the type of diet (Xiao et al., 1991b). The majority of activity for the hydrolysis of OA in the rumen against the toxin was attributed to rumen protozoa (Kiessling et al., 1984). The pattern of hydrolysis of OA was the same in the reticulum and omasum. In some cases OA is esterified to OC (Galtire and Alvinerir, 1976)

In addition to OA being converted to Oα and possibly OC, a small percentage of absorbed OA is converted into hydroxyochratoxin A. After incubation of ochratoxin A with rat liver microsomes and NADPH, 90% of metabolites corresponded to 4-hydroxyochratoxin A. This metabolism was inhibited by carbon monoxide and metyrapone (Støren et al., 1982). Størmer and Pederson (1980) demonstrated that liver microsomes from humans, pigs, and rats were also capable of metabolizing OA in the presence of NADPH to (4R)- and (4S)-hydroxyochratoxin A. These observations suggested that microsomal hydroxylation occurs in the cytochrome P-450 system. The rate of formation of OA-OH increased in microsomal preparation from phenobarbital-treated animals (Hansen et al.,

1982; Galtier, 1991; Størmer, 1992). Fink-Gtrmmels et al. (1993, 1995) demonstrated that metabolite formation was induced in hepatocytes obtained from phenobarbital (PB) pretreated animals and increased in a time dependent manner. *In vivo* studies demonstrated that 1 to 1.5% of orally administered OA was excreted in the urine as (4R)-hydroxyochratoxin A and 25 to 27% as Oα (Støren et al., 1982). Størmer et al. (1983) reported that rabbit liver microsomes in addition to producing the 4R and 4S isomers also produced 10-hydroxyochratoxin A. Metabolism of OA to the hydroxylated form increased in phenobarbital-treated mice and rats and, as a result, its toxicity decreased (Størmer et al., 1983; Hutchinson et al., 1971), whereas Creppy et al. (1983a,b, 1986) reported that OA and the 4R-hydroxy form of OA were equally toxic to yeast and that both inhibited phenylalanyl-tRNA synthetase to a similar degree. The lower toxicity of the hydroxy form of OA to mammals may possibly be attributed to a faster rate of clearance in the urine or feces compared with that of OA. Castegnaro et al. (1989) demonstrated a difference between two strains of rats in their ability to form 4-hydroxy-OA and that this difference was associated with a strain difference in a genetic polymorphism for debrisoquine 4-hydroxylation. They hypothesized that there is a positive association between the ability of animals to hydroxylate OA and the incidence of OA-induced tumor incidence. These results suggest that there is a relationship between the degree of hydroxylation of OA and the susceptibility of rats to the nephrotoxic effects of OA and, therefore, prominent toxicological effects might be linked to the biotransformation processes.

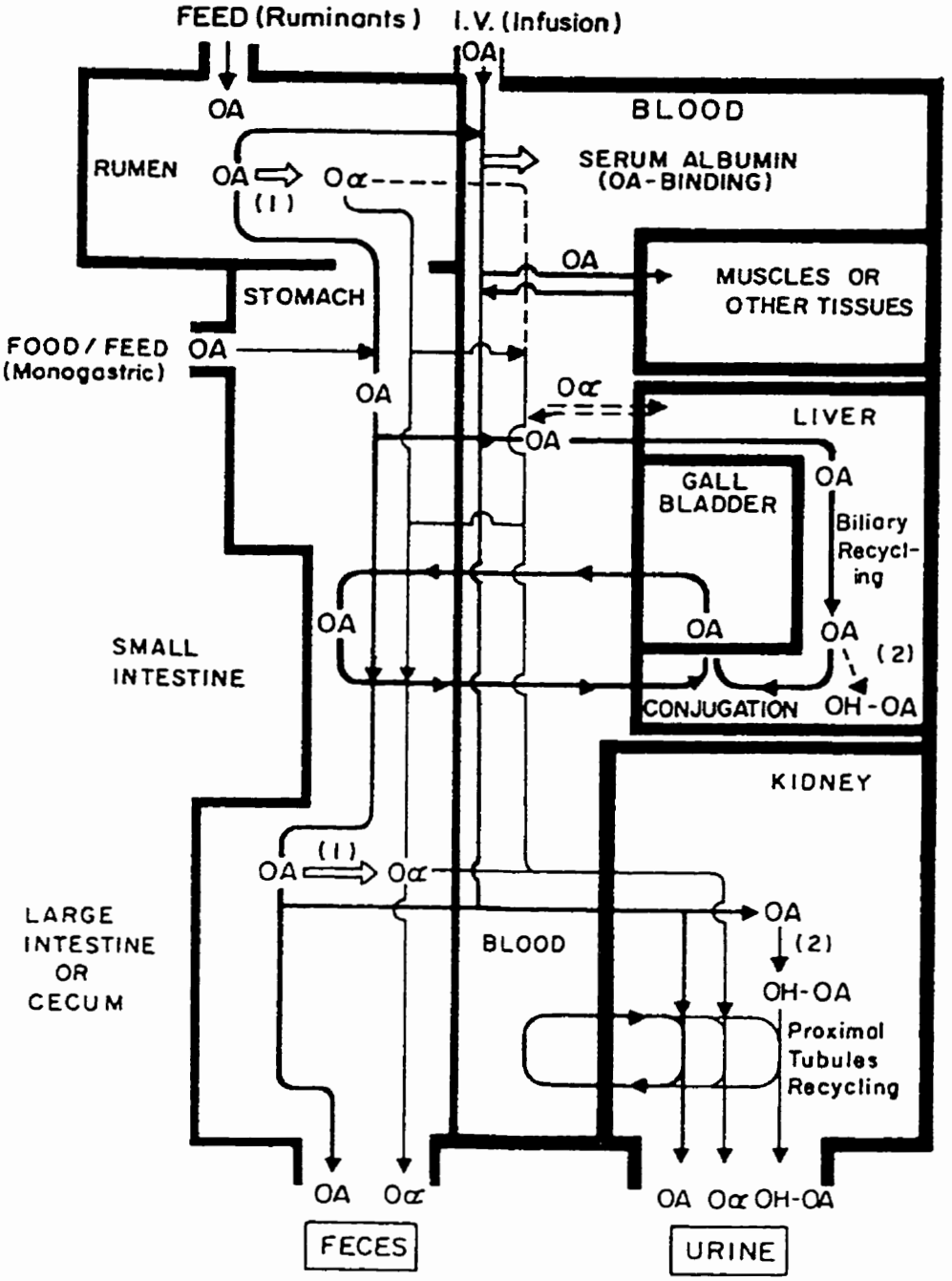
6. Pharmacokinetics of Ochratoxin A, its Analogs and Metabolites.

Pharmacokinetics is the study of the time-course changes in the concentration of drugs or chemicals and its metabolites in the body (Shargel and Yu, 1985). Pharmacokinetic patterns of absorption, distribution, metabolism and excretion of OA and its metabolites for several species of animals have been reviewed (Kuiper-Goodman and Scott, 1989; Marquardt and Frohlich, 1992). OA is absorbed from the alimentary tract, mainly the stomach and the small intestine through the intestinal mucosa into blood. The high binding affinity of OA to plasma albumin and its recycling in the bile and kidney contributes to its long half-life in animals (Marquardt and Frohlich, 1992). OA is hydrolyzed to its nontoxic form, Oα, by microorganisms in rumen, cecum and large intestine or to a much lesser degree by carboxypeptidase A in the small intestine. Ochratoxin A is also hydroxylated to hydroxy form in the liver by the P-450 system. The toxin is excreted mainly through the bile, urine, and feces (Krogh, 1983). A schematic for disposition of OA, Oα, and OA-OH in the ruminant was described by Marquardt and Frohlich (1992) in Figure 4. The bioavailability of OA, which is the amount of OA reaching the systemic circulation, therefore, affect its toxicity of OA (Marquardt and Frohlich, 1992).

6.1 Absorption:

Animals fed with OA contaminated feedstuffs is absorbed primarily from the stomach (Galtier, 1978; Kumagai and Aibara, 1982; Roth et al., 1988). Studies using immunohistochemical stains suggest that absorption of OA takes place maximally in the

Fig 4. Schematic for disposition of ochratoxin A (OA), ochratoxin alpha (Oα) and hydroxylated OA (OA-OH) in the ruminant. 1, represents hydrolysis of OA to Oα; 2, hydroxylation of OA to OA-OH. Recycling of OA and possibly Oα and OA-OH in liver and kidney are indicated by closed loops. Adapted from Marquardt and Frohlich (1992).


FEED (Ruminants)
I.V. (Infusion)
OA
BLOOD
RUMEN
OA
(1)
Oα
SERUM ALBUMIN
(OA-BINDING)
OA
MUSCLES OR
OTHER TISSUES
STOMACH
FOOD / FEED
(Monogastric)
OA
OA
Oα
LIVER
OA
GALL
BLADDER
OA
Biliary
Recycl-
ing
SMALL
INTESTINE
OA
OA
OA
(2)
CONJUGATION
OH-OA
KIDNEY
(1)
OA
Oα
OA
LARGE
INTESTINE
OR
CECUM
BLOOD
(2)
OH-OA
Proximal
Tubules
Recycling
OA Oα
OA Oα OH-OA
FECES
URINE

esophagus, duodenum, and to a lesser degree in the jejunum (Lee et al., 1984), confirming previous finds that OA absorption occurs in the upper portion of the gastrointestinal tract. Intravenous administration of OA indicated that jejunal absorption of OA occurred even when the jejunual lumen has a lower concentration than plasma (Kumagai, 1988). This was attributed to the low mucosal pH present in the jejunum. There was no uptake of OA by the ileal mucosa membrane. Other studies on pigs, rabbits, and chickens also show the rapid absorption of OA from the gastrointestinal tract (Galtier et al., 1981, Lee et al., 1984). The absorption site of OA in the ruminant is not clear as the three prestomachs in the ruminant are unique, differing from the gastrointestinal tract of monogastric animals (Marquardt and Frohlich, 1992). No report has clearly established the influence of the rumen on the absorption of OA.

The primary absorption route of OA was suggested to be the portal vein although the contribution of the lymphatic route could not be excluded when a low dose of OA was given (Kumagai and Aibara, 1982). Ochratoxin A also seems to be absorbed in a passive manner from the gastrointestinal tract in the nonionized or partially ionized form (Kumagai, 1988). OA exists in both ionized and non-ionized forms in aqueous medium because of the partial dissociation of the phenolic hydroxy group in the dihydroisocoumarin ring (Chu et al., 1972). The proportion of the two forms can be calculated from its pKa value (7.1). The pH of digesta should therefore affect the rate of absorption of OA with the rate being faster in those sections of the gastrointestinal tract that have a low rather than a high pH (Marquardt

and Frohlich, 1992).

Regarding the absorption of OA, facilitated diffusion or mediated transport had been assumed to be the main mechanism of intestinal transfer of this weak electrolyte. Kumagai and Aibara (1982) and Kumagai (1985) demonstrated that the absorption of OA in the jejunal segment was linearly related to the concentration of OA in blood plasma and that it was absorbed even when its level was higher in plasma than in the jejunal lumen. The jejunal uptake of OA increased with a decrease in pH of the medium, with the increase in uptake coinciding with an increase in the proportion of OA present in the non-ionized form (Kumagai, 1988). OA uptake by the intestinal mucosa was found to be Na^+ dependent, but the flux of OA from mucosa layer was found to be Na^+ independent (Kumagai, 1988). These results suggested that diffusional movement of organic anion plays a major role in OA absorption. In addition, a concentration gradient of the non-ionized form of OA from the jejunal lumen to the blood and lymph may occur even though the concentration of OA is higher in the blood plasma than that in the lumen since the pH value is different between plasma (pH 7.4) and gastric juice (pH 1.25). Therefore, the nonionized form of OA which occurs at the low pH of the stomach can be readily transported into the blood whereas the ionized form as occurs in the plasma cannot be transported back into the stomach. The binding of OA to plasma albumin also helps in the maintenance of a concentration gradient of OA between plasma and body tissues (Kumagai and Amara, 1982).

6.2 Tissue Distribution:

The distribution of OA labeled with ^{14}C in rats and day-old chicken tissues indicated the highest level of radioactivity was in serum, kidney and liver (Chang and Chu, 1977; Frye and Chu, 1977). Studies on the distribution of ^{14}C OA in Japanese quail indicated that after dosing with radio-labeled OA, it was detected in almost every organ and tissue: blood, myocardium, liver, proventriculus, gizzard intestinal mucosa, pancreas, kidney, ovary, egg yolk, brain, red and white muscle, lung, and the uropygial gland (Fuchs et al, 1986, 1988a,b). Oral administration of ^{3}H labeled OA to rats indicated that the distribution in tissue of the specific label in decreasing order was: the intestinal content, lung, liver, kidney, heart, fat, intestine and testes with the lowest specific activity, occurring in muscles, spleen and the brain (Kane et al., 1986). The report suggested that the toxin is distributed in relatively high concentrations in its target organs, the liver and kidney. In another study it was shown that the concentration of OA in the blood was much higher than that seen in other tissues (Mortensen et al., 1983).

OA was shown to cross the placental barrier of pregnant mice that were intravenously administered ^{14}C-labeled OA on day 9 of pregnancy (Appelgren and Arora, 1983). OA was also detected in serum, muscle, skin, kidney, liver, heart, lung, uterus, placenta, and the fetus of pregnant rats administered labeled OA (Ballinger et al, 1986) and in kidney, liver, muscle, placenta and the fetus in pregnant pigs dosed with OA (Patterson et al, 1976). The results indicated that the principal target organs were the kidney and liver, as they had the highest

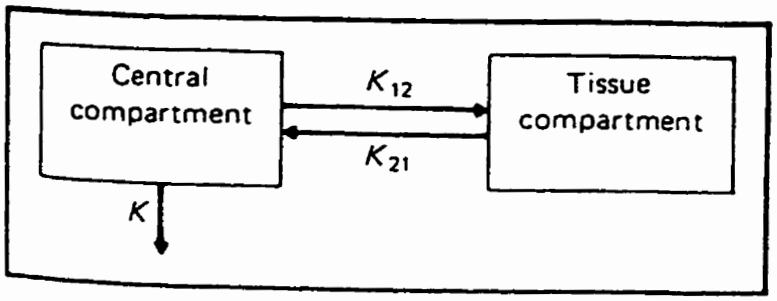
concentrations of OA in the body and that OA can cross the placental barrier into the maternal circulation thereby affecting the fetus. In summary, the results of the above studies demonstrated that OA is taken up by all tissues in the body but to varying degrees. The highest concentration is found in the blood, followed by the primary target tissues, the liver and kidney.

6.3 Kinetics of tissue distribution:

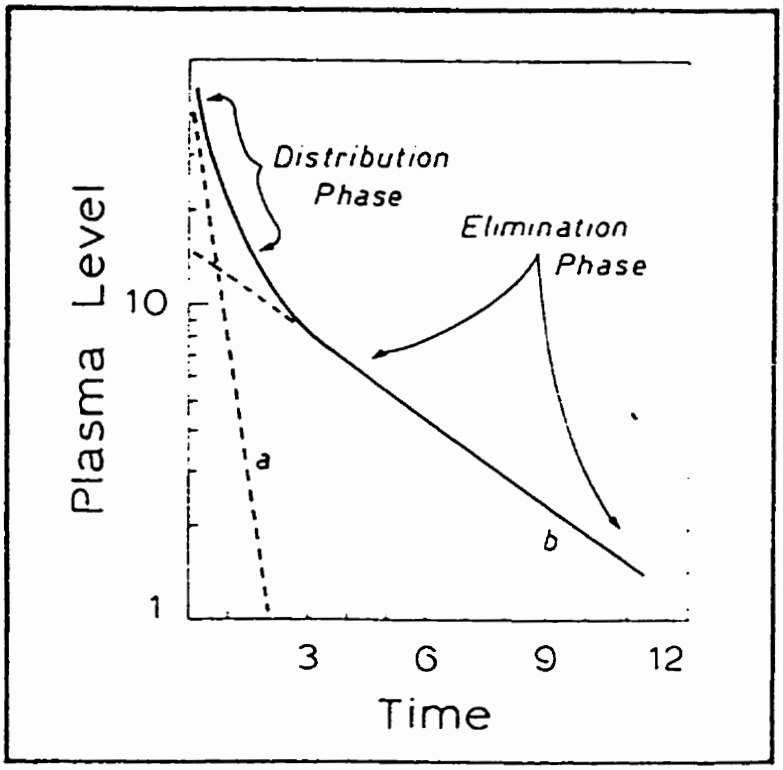
Ochratoxin A have demonstrated a plasma level-time curve by assuming first-order transfer of OA following a single intraveneous dose into a two compartment open model which was described by Galtier et al. (1979) and Hageberget et al. (1989). In the case of a two compartment model , one compartment, known as the central compartment, represents the blood, extracellular water, and highly perfused tissue; this compartment is rapidly diffused with toxin. A second compartment, known as the tissue compartment, contains tissues which equilibrate more slowly with the toxin (Shargel and Yu, 1985) (Fig 5).

Ochratoxin A is rapidly absorbed across the intestinal mucosa into the bloodstream and distributed to various tissues (Nel and Purchase, 1968). Typical semilogarithmic plots of the concentration of OA and its metabolites in blood following single intravenous injection of the different compounds declined in a biexponential manner, which suggests that the distribution followed a two compartment open model (Galtier et al., 1979; Hagelberg at al., 1989). The initial distribution phase ($t1/2\alpha$, distribution half-life) demonstrates that this is a rapid process since the half-lives are relatively short. The

Fig 5. I, Two compartment open model, iv injection. II, Plasma level-time curve for the two compartment open model (single iv dose) according to Shargel and Yu (1975).


Central
compartment
K_{12}
K_{21}
Tissue
compartment
K

I


Distribution
Phase
Elimination
Phase
Plasma Level
10
1
a
b
3
6
9
12
Time

II

distribution half-life of OA was 1.8 hour in cattle (Sreemanarayana et al., 1988), 2.1 h in rats (Galtier et al., 1979), and 1.9 h in rabbit (Galtier et al., 1981).

In ruminant calves OA was shown to be absorbed rapidly by a first order process after oral administration, followed by a rapid monoexponential decline and then to a distribution phase (Sreemannarayara et al., 1988). The single protracted secondary peak suggested that this was attributable to enterohepatic recycling of OA (Sreemannarayara et al., 1988; Roth et al., 1988; Fuchs et al., 1988a,b). Studies on cattle indicate that the maximum plasma concentration of OA (0.44 μg/ml) is obtained 2 to 4 h after a single oral dose of 1 mg OA/kg body weight (Sreemannarayana et al., 1988).

6.4 Excretion:

Elimination of OA involves both biotransformation and excretion. Elimination of OA can be expressed in terms of either the elimination rate constant (Ke) or the elimination half-life ($t1/2\beta$). The elimination half-life of OA was 77.3 h in young cattle (Sreemannarayana et al, 1988), 8.2 -10.8 h in rabbits (Galtier et al., 1981), and 55.9 h in pregnant mice (Ballinger et al, 1986). The elimination half-life of OA from blood for rats was 120 h for iv injected OA and 170 h for peripherally administered OA (Hagelberg, et al., 1989) and 55 and 56 h for either oral or iv injected rats (Galtier, et al., 1979; Ballinger et al., 1986). Large differences in the elimination half-life of OA other species after oral or iv administration have being reported with the values being 72-150 h for the pig (Galtier et al., 1981; Hagelberg et al., 1989), 24-48 h in mouse (Fukui, et al., 1987; Hagelberg, et al., 1989), 6.7-

12 h in quail (Hagelberg et al., 1989), 3.0- 4.1 h in chickens (Galtier et al., 1981), 0.7- 0.8 h for fish (Hagelberg et al., 1989), 32.8 and 28.7 h in the placenta and serum of mouse following ip administration of a 5 mg/kg dose (Fuhui et al., 1987), and 127 h for injected rats (Breitholtz-Emanuelsson, 1995). The longest elimination half-life was 510 h for monkey (Fuchs and Hults, 1992).

The elimination rate constant of OA in the rat when administered orally is 0.028/h for oral dose and 0.039/h when administered by an intravenous injection (Galtier et al., 1979). Studies by Sreemannarayana et al. (1988) in young cattle injected intravenously with OA demonstrated that about 7% of the OA was excreted unchanged in urine and 11% in feces indicating that ochratoxin A is excreted into the bile. The prolonged elimination half-life obtained for OA in animals including rats seems to be influenced by the high binding affinity of OA to plasma albumin, and the significant reabsorption and redistribution of OA in the kidney and via enterohepatic circulation (Stein et al., 1985; Sreemannarayara et al., 1988; Hagelberg et al., 1989; Fuchs and Hult, 1992). Ochratoxin A administered to rats orally appeared in the bile, with maximal excretion taking place within the first 6 and 24 h later was higher when the toxin was administered into the duodenum than when given into the stomach (Fuchs et al., 1988c). Biliary recycling is therefore significant in terms of the bioavailability of OA. The secondary peak that was seen in the pharmacokinetic profile in ruminant calves given an oral dose of OA suggests that the toxin is excreted into the intestinal tract via the bile and is reabsorbed from the intestine with reabsorption acting as

a second small dose of OA (Sreemannarayana et al., 1988). Cholestyramine (CHA), which blocks enterohepatic cycling, eliminates the secondary peak of OA, thereby changing the elimination profile of OA (Madhyastha et al., 1992b). These findings further support the proposal that biliary recycling of OA occurs (Roth et al, 1988). Secretion and reabsorption of OA by the renal proximal tubule has been investigated in rats. Blocking the renal secretion decreased the renal clearance of OA and increased the nephrotoxicity of the toxin (Stein et al, 1985). The results also indicated that secretion of OA via organic anion transport is an important mechanism by which the kidney handles the toxin, and renal tubular secretion and reabsorption may facilitate the residual persistency of OA in kidney. The mechanism of OA transport in the kidney has been confirmed to be mediated via the renal organic anion transport system (Sokol et al., 1988). Therefore an efficient reabsorption of OA by the renal tubules of the kidney greatly facilitates reabsorption of OA into the plasma. This recycling may be responsible for gross change in the kidney, including progressive degeneration of the renal tubules in rats (Albassam et al., 1987), pigs (Szczech et al., 1973), and chicken (Elling et al., 1975, 1979).

OA is distributed in the free and bound forms in the liver, kidney, spleen and other tissues and organs with most of the OA being bound to the serum albumin (Nel and Purchase, 1968; Chu, 1971; Lee et al., 1984; Hagelbery et al., 1989). Human serum albumin had a much higher affinity for OA than did α1-, α2-, β- and γ-globulins (Uchiyama and Saito, 1987). Binding of OA to low molecular weight plasma constituents (around 20,000)

may be relevant to its predominant nephrotoxic effects in mammals (Stojkovič et al., 1984). The high binding affinity of albumin for OA retards its elimination and thereby prolongs its half-life (Chu, 1971; Krogh, 1983). Plasma concentration of OA dropped to 0.5 μg/ml 10 min after injection of OA (2.2 mg/kg) into an albumin-deficient rat, while it remained at 50.0 μg/ml for more than 90 min in the normal rat (Kumagai, 1985). The concentration of OA in plasma was higher than that in the bile and urine in normal rats, while the opposite result was obtained in the albumin-deficient rat (Kumagai and Aibara, 1982; Kumagai, 1985). The bioavailability of OA in the calf fed with milk immediately after dosing was 3-7 times higher than that in the non- fed calf, suggesting that binding of OA to milk protein will increase its toxicity (Sreemannarayana et al., 1988).

6.5 Clearance of ochratoxins and metabolites formed:

Essentially no studies have been carried out on the clearance of OA from animals. Data from Madhyastha et al. (1992a) demonstrated in rats that approximately 70% of the total OA plus Oα was excreted in the feces with the balance being in the urine. They also demonstrated that up to 38% of the OA plus Oα that was excreted in the urine plus feces was excreted as Oα with more than half of that being excreted in the urine. They suggested that hydrolysis of OA by the microorganisms in the digesta were responsible for most of the production of Oα and that it was reabsorbed into the blood and then excreted in the urine. Støren et al. (1982) reported that 13% of the total OA plus OA-OH that was excreted in the urine was as OA-OH 4(R).

Several metabolites or conjugates in addition to OA-OH and Oα are formed in the rat from OA and OB, including Oβ [see Marquardt and Frohlich (1992) for a review] and the lactone-opened form of OA (Xiao et al., 1996a). Detailed research on the nature of other breakdown products and their route of excretion have not been published.

7. Summary and Conclusions

The ochratoxins are a family of mycotoxins which have structurally related derivatives. Included are OA, OB, OC, OA-OH, Oα, Oβ and OP-OA. OA, OC and OP-OA are highly toxic while Oα, Oβ and OA-OH are metabolites of the parent compounds and are either non-toxic or have a low degree of toxicity. Preliminary data suggest that there are many metabolites of the ochratoxins. Several methods have been developed for the analysis of the ochratoxins with one of the most widely used being HPLC. Also several methods been developed for the confirmation of the ochratoxins in tissues. Most of these procedures are accurate but are time consuming and expensive, and in some cases lack the desired level of sensitivity.

The ochratoxins are produced by fungi, especially *A. ochraceus* and *P. verrucosum*. There production is influence by several environmental factors especially substrate, temperature and moisture content of the substrate. The two species of OA producing fungi are widespread throughout the world with *A. ochraceus* being found mainly in tropical and subtropical areas while *P. verrucosum* is found mainly in temperate regions. OA is also

found in grain, pork products, human blood and breast milk. It is especially prevalent in Canada, Northern Europe and the Balkan countries. OA is a carcinogen, hepatotoxin, and a nephrotoxin. Several different modes of action of OA have been suggested. OA is hydrolyzed mainly by intestinal microorganisms to the nontoxic Oα, or is hydroxylated by the P-450 system to different forms of OA-OH. It is also converted into OP-OA in the body which is similar in toxicity to OA. OA is absorbed passively mainly from the stomach in its nonionized form. The half-life of OA is long while that of its metabolites is unknown. Factors that tend to prolong the half-life of OA is its ability to bind strongly to serum albumin, and its high degree of enterohepatic and kidney recycling. Very little is known about the route and degree of clearance of OA and its metabolites from the body. The objectives of this study, as indicated in the "General Introductions", was to develop new and simple methods for the confirmation of the ochratoxins in tissues, blood, bile and urine samples, and to provide comprehensive data on the half-lives of the different ochratoxins and to establish the nature of their clearance.

MANUSCRIPT I

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 Agricultural and Food Sciences, University of Manitoba

Abstract

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 more than 95% when the sample was incubated for a period of more than 12 h at 20°C in the presence of 6N 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.

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, 1965a,b). The family of ochratoxins includes several structurally related analogs 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., 1965a; 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 (less than 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 a 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 contaminates would coelute with both OA and its ester, OC. 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 base 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.

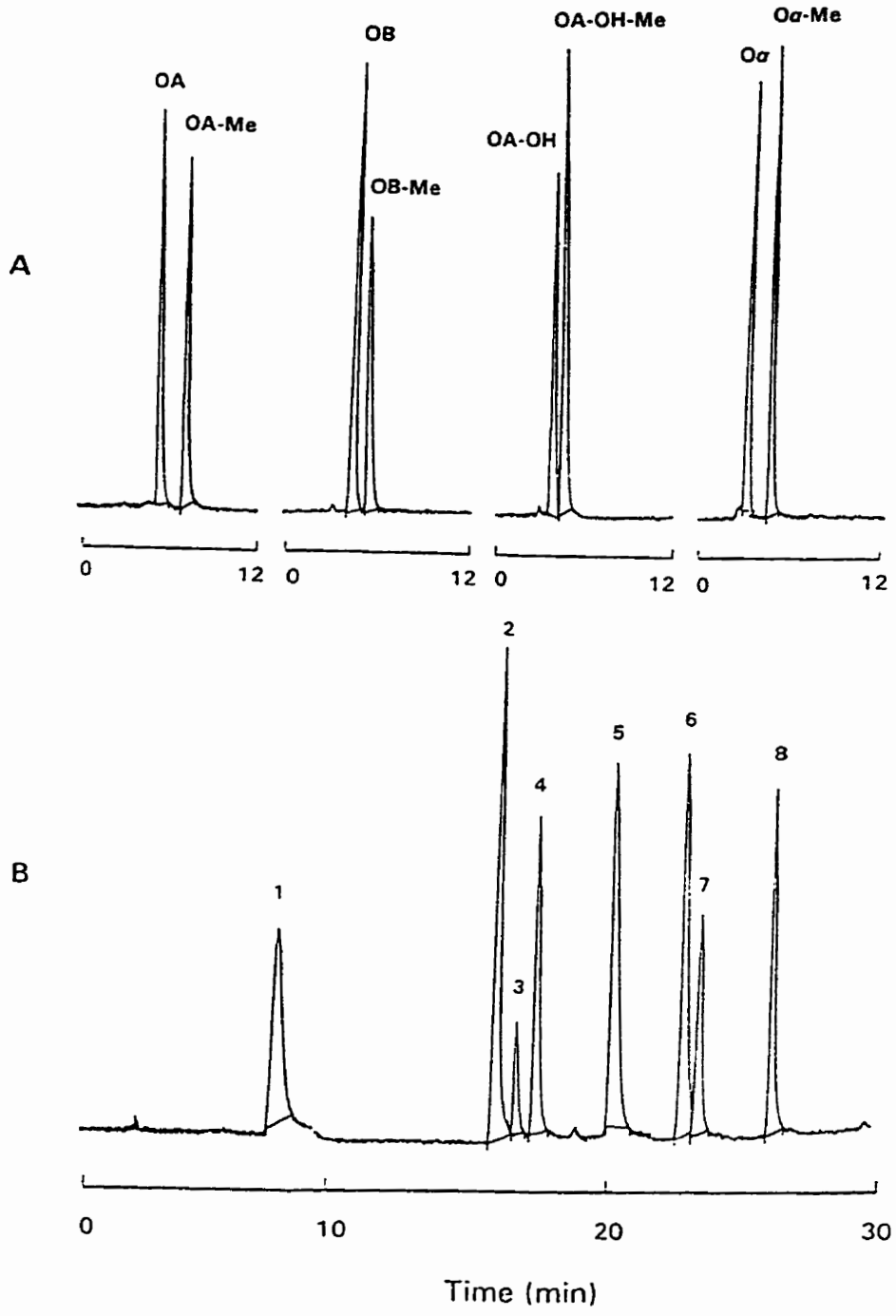
Materials and Methods

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 isopropanol (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 (Fig 6A). 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 (Fig 6B). Isocratic separation was used for samples that did not contain extracts from animal tissues (experiments 1- 3 and 5)

Fig 6. 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: 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: 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 further detail.


A
OA
OA-Me
OB
OB-Me
OA-OH
OA-OH-Me
Oα
Oα-Me
0
12
0
12
0
12
0
12
B
1
2
3
4
5
6
7
8
0
10
20
30
Time (min)

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 approximately 10% of the mean for replicate analysis. This limit was at least 10-fold lower than the lowest value reported in this study.

Experiment 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 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 after incubated for 1, 3, 6, 12, 18, and 24 h (Table 2). 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 (Fig 2). OA in methanol (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 6N HCl; D, 450 MeOH, 25 6N 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 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 toxin /ml methanol) (Table 3). The total amount 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 saline, respectively. The bile was collected through a bile duct 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 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 13,000 xg and the 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/0.1M $MgCl_2$, 6 ml of $CHCl_3$, and 100 μl of OA for the spiked samples (5 μg/ml in methanol) and 100μl methanol for 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 1,300xg 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 distilled water was added into each tube and mixed well, and the sample was centrifuged at 1,300xg 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, 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 250μg of OA/ml 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 12N HCl. Therefore, for MS analysis, high concentrations of OA, OB, OA-OH, and Oα were converted into their esters using 12N HCl for 24 h with the conversions being 99, 98, 100 and 26%, respectively. The samples (20 ml; 19 ml methanol and 1 ml 12N HCl) were extracted with 20 ml chloroform and 100 ml 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.

Results

The results from experiment I 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 more than 60% conversion being completed within 6 h and 87% at 24 h (Table 2, 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 is formed in nonacidified methanol (data not shown). In the second experiment, the volumes of methanol relative to water (Fig 8C, D), the concentration of acid (Fig 7D-G) and the incubation time (Fig 7 upper frames, 6 h); degree of conversion of OA into OA-Me were 53 and 94% after 6 h more than a month traces of OA-Me ester is formed in non-acidified methanol (data not shown at 24 h) affected the degree of conversion of OA into OA-Me and Oα. The 86 and 96% after 12 h when the concentration of acid in the aqueous portion of the mixture were 2 and 6N HCl and the percents methanol (v/v) in the sample were 85 and 95%, respectively (C vs D). The total amount 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.

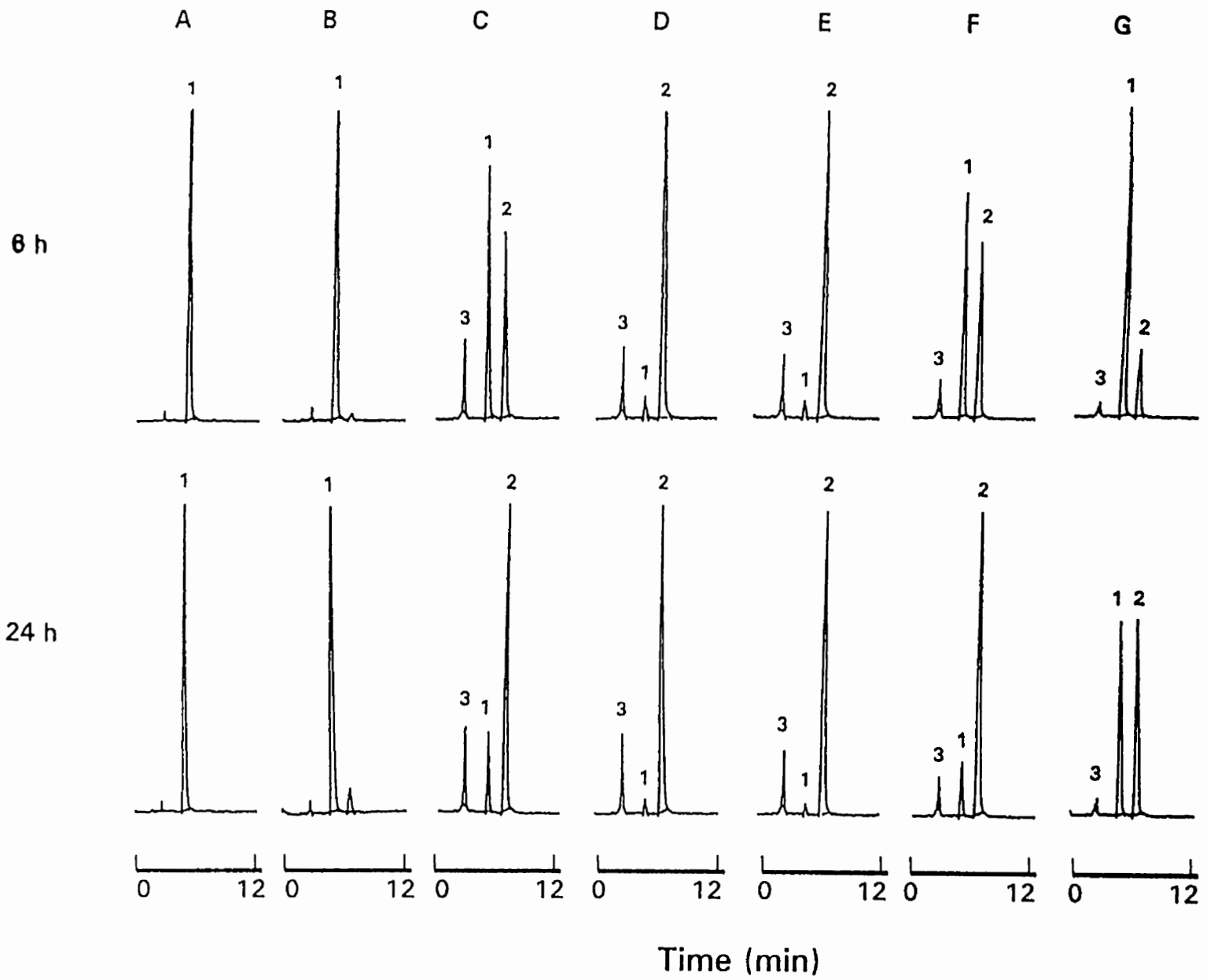
Table 2. Conversion of ochratoxin A into the methyl ester in the presence of methanol acidified and incubated for different time periods in experiment 1.

	Treatment [1]		Conversion of OA (%) into new product [2]					
	% MeOH (v/v)	N of HCl	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

[1] 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).

[2] The elution time for OA was 4.45 min while 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 analysis.

Fig 7. Chromatography of ochratoxin A 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 (μl) of added acid or water were 0, 75, 75, 25, 25, 25 and 25, respectively with the total final volume being 500 μl (see Materials and Methods, experiment 2 for further detail). The elution times for peaks 1, 2 and 3, respectively were 4.55, 5.99 and 2.47 min, respectively, which corresponded to elution times for OA, OA-Me and Oα in the standard chromatograms (Fig 6A).


A
B
C
D
E
F
G
6 h
24 h
1
2
3
0
12
Time (min)

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 3 (experiment 3). The total amounts of HCl in the two samples containing 2 or 6N 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 more than 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 (Fig 8, S and T). The same samples when incubated with 2 N (bile) or 6N (liver or kidney) HCl and methanol (85% for bile or 95%

Table 3 The conversion of ochratoxins (OA, OB, Oα and OA-OH) into their methyl esters when acidified with 6N HCl with or without water and incubated for 6 and 24 hours at 25°C in experiment 3.

Treatment				Concentration of ochratoxins (μg/ml) and new product conversion (%)					
				6 h			24 h		
Toxin[1]	% MeOH (v/v)	N of HCL added	volume of HCl added (μl)	Toxins		Conversion (%)	Toxins		Conversion (%)
				OA	P[2]		OA	P[2]	
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	0	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	11.2	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

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

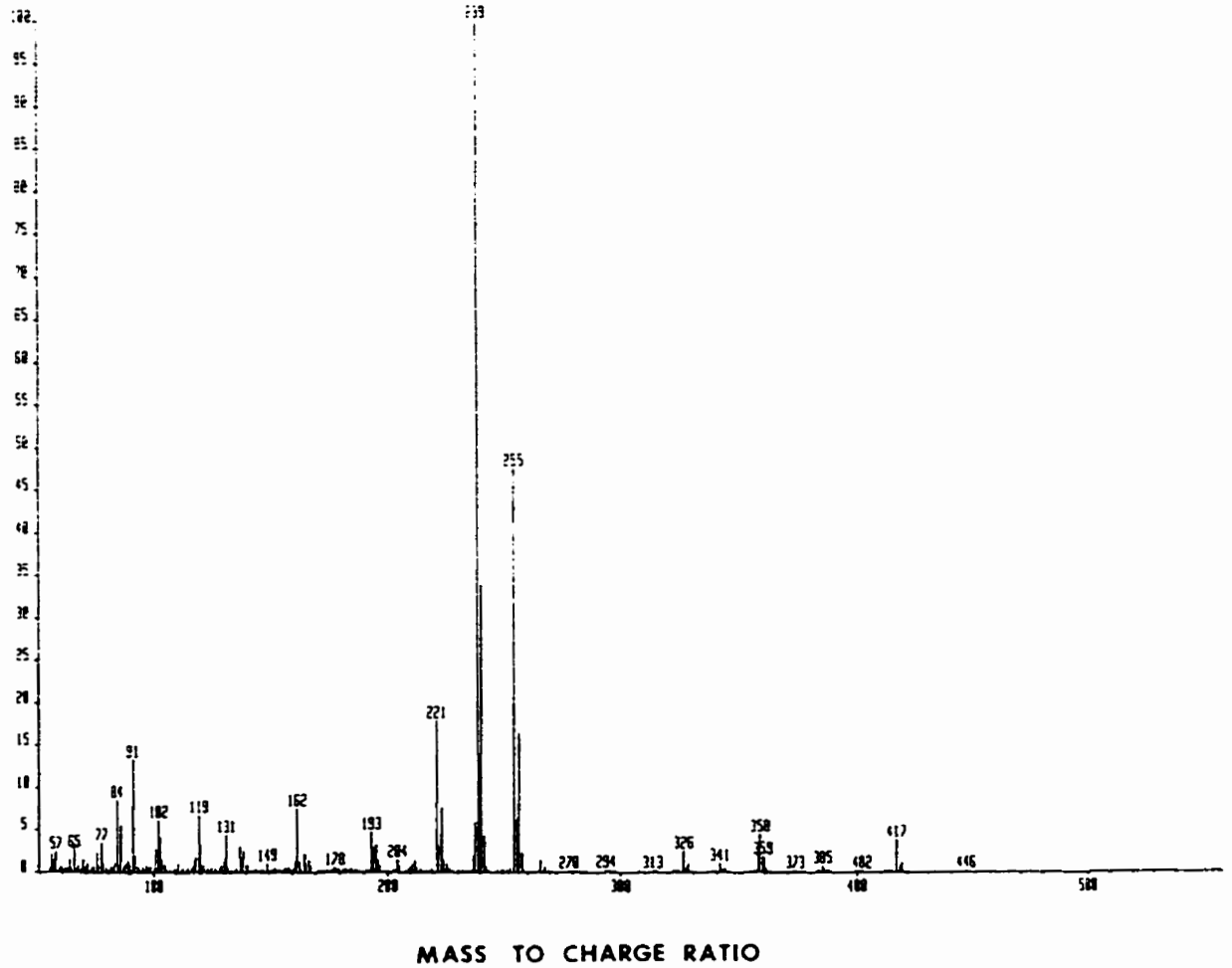
for liver or kidney spiked (S+A) and treated ey) had nearly quantitative conversion of OA into OA-Me except for (T+A) bile which, respectively, contained 11 and 25% OA and 89 and 75% OA-Me as determined from peak areas (Fig 8, S+A and T+A). The apparent reduced conversion of OA to OA-Me in the two bile 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 3). Although the concentration of acid in the bile compared to the liver and kidney samples were different (2 or 6N HCL) the total amount of added acid were the same (Table 3). The percent conversions of OA into OA-Me in the 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.

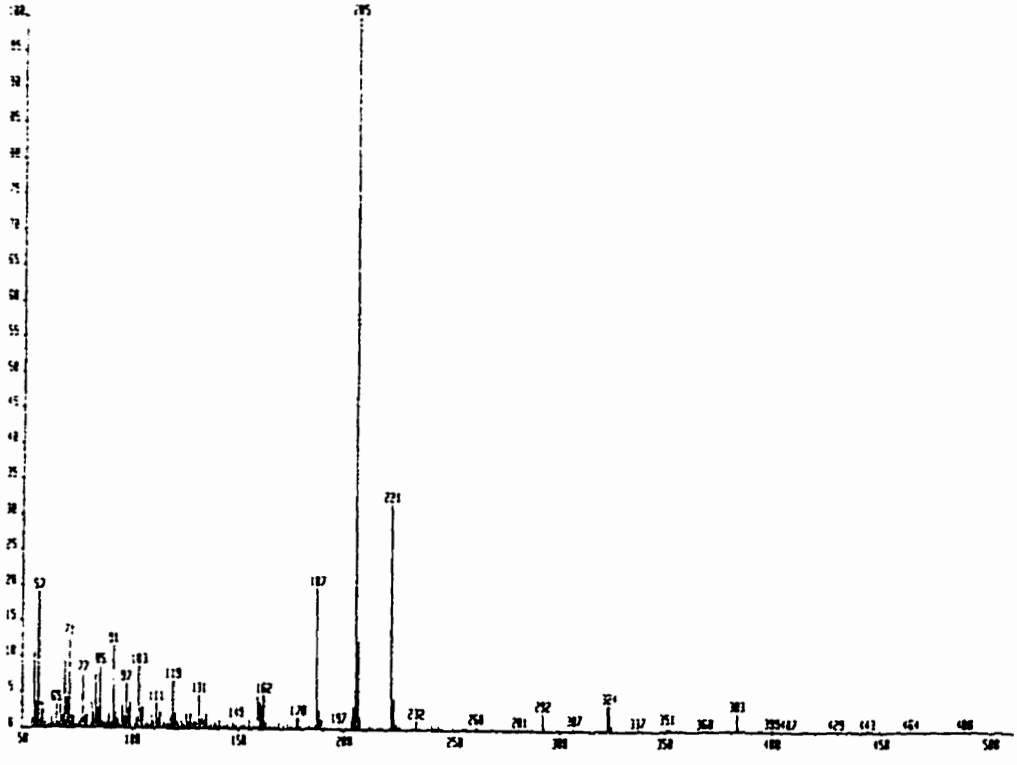
Fig 8. Chromatography of ochratoxin A (OA) from tissues including bile, liver and kidney tissues of rats acidified (S+A or T+A) or not acidified (S or T) with HCl and incubated in methanol for 12 h at 25°C. The tissues were obtained from rats not treated with OA and spiked with OA (S) or from treated rats injected intravenously (bile) or intraperitaneally (liver and kidney) with OA (T). The methanol in bile was 85% while that in liver and kidney was 95%. The corresponding concentrations of HCl in the aqueous solution (tissue + added acid) were 2 or 6N with the total amounts of acid being the same for all samples. The procedure for extraction and incubation of the samples were as described in experiment 4 of Materials and Methods.

The percent conversion (more than 98% except for Oα) and recovery as estimated from relative peak areas (more than 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 pattern 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 der 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) (Figure 9).

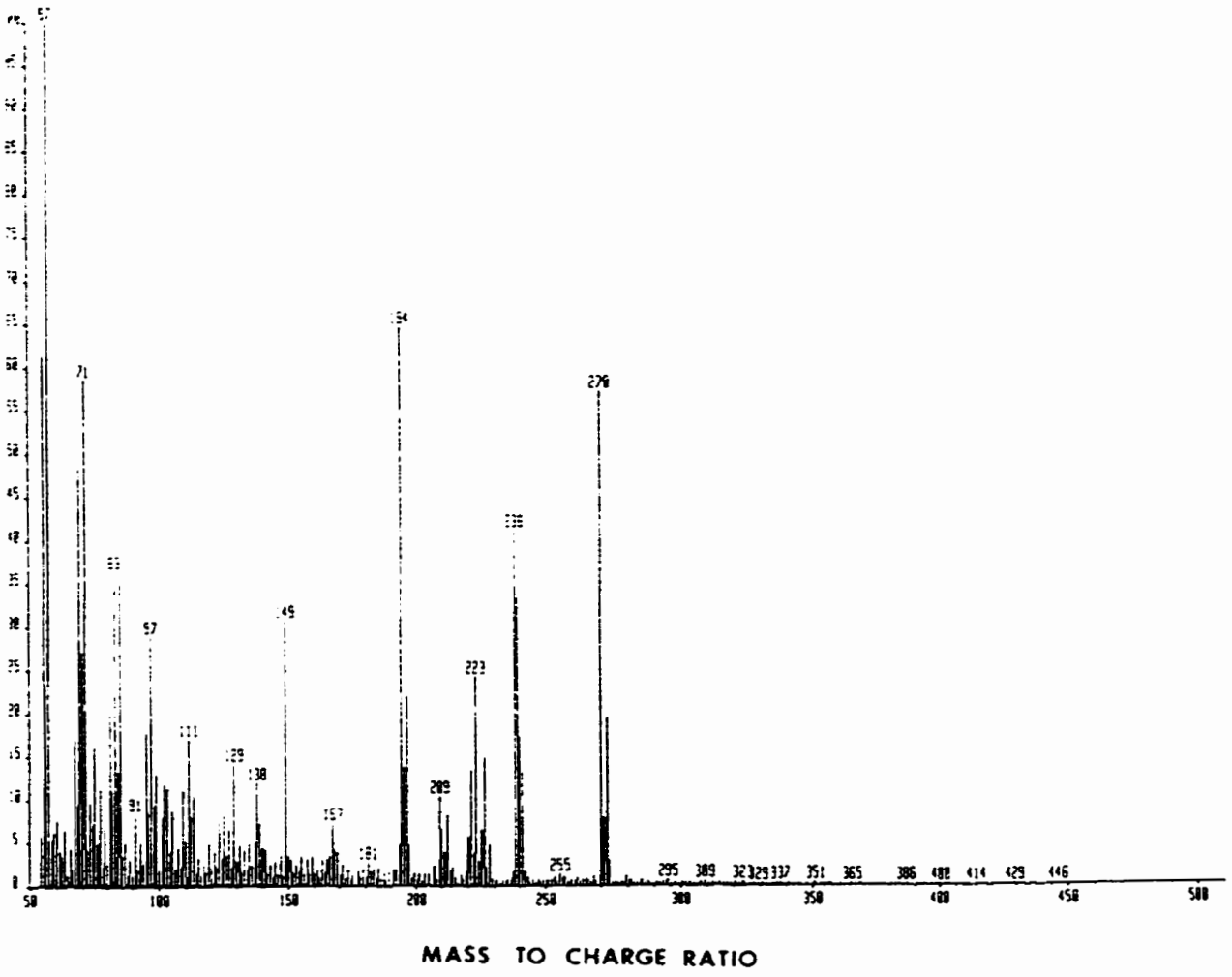
Fig 9. Mass spectrometry of: I, Ochratoxin A methyl ester; I, Ochratoxin B (OB) methyl ester; III, Ochratoxin alpha (Oα) methyl ester; IV, Hydroxychratoxin A (OA-OH-Me) methyl ester.



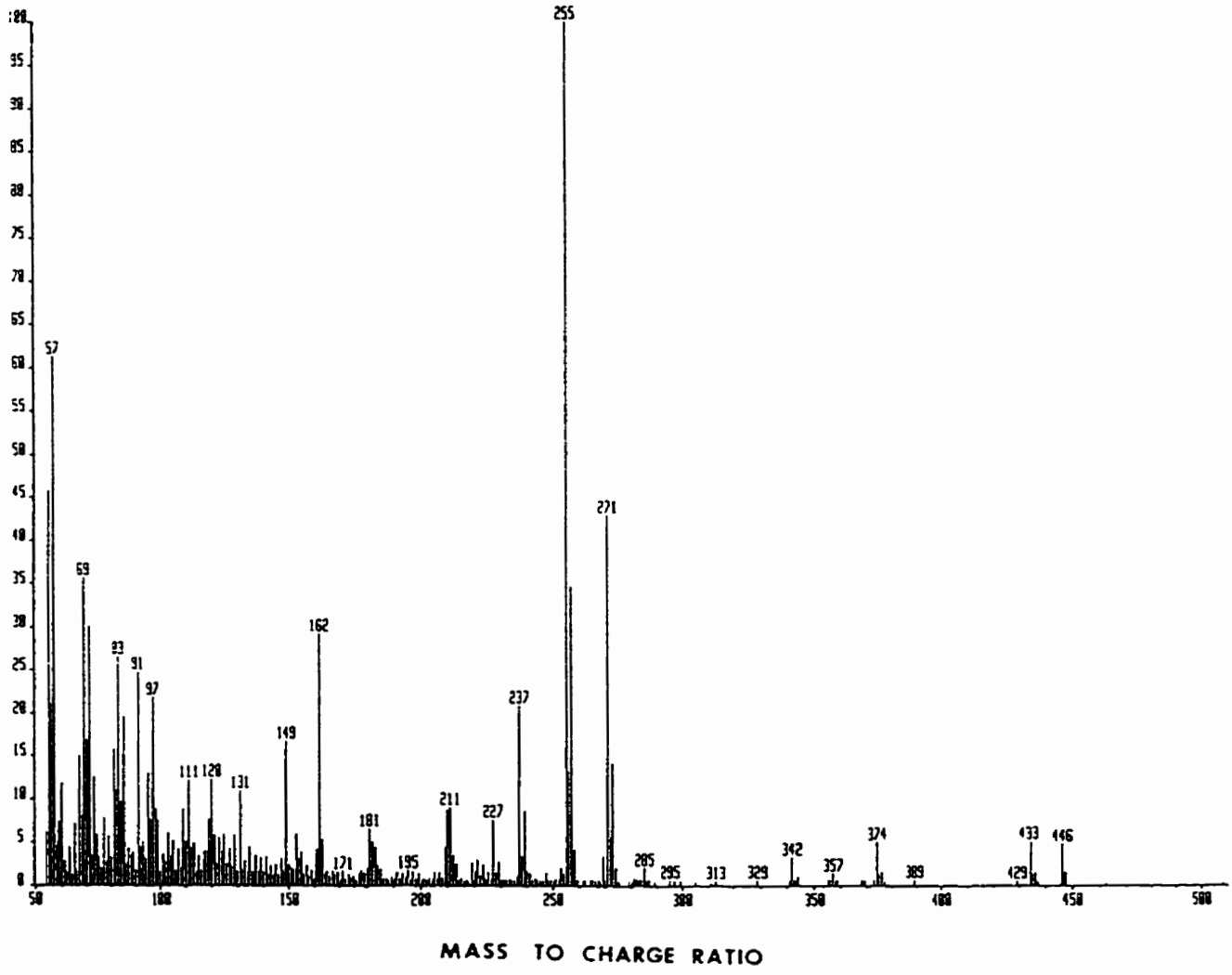
I, Ochratoxin A methyl ester.



II, Ochratoxin B (OB) methyl ester.



III, Ochratoxin alpha (Oα) methyl ester.



IV, Hydroxychratoxin A (OA-OH-Me) methyl ester.

Studies were also carried out with ethanol rather than methanol. Similar trends were obtained except the percent conversion of the different ochratoxins to their ethyl esters were lower than those obtained with methanol. The 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 ochratoxins 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.

MANUSCRIPT II

Metabolites of The Ochratoxins in Bile and Urine of Rats

S. Li, R. R. Marquardt, and A. A. Frohlich

Department of Animal Science, University of Manitoba,

Abstract

The objectives of this study were to determine the metabolic profiles of the ochratoxins in the bile and urine of rats injected with several different forms of ochratoxin including ochratoxin A (OA), lactone opened OA (OP-OA), ochratoxin B (dechlorinated OA, OB), hydroxy OA (OA-OH), and ochratoxin α (hydrolyzed OA, Oα). The HPLC gradient used in this study was able to clearly resolve the different ochratoxins and their metabolites without the need for a cleanup procedure. A total of more than 20 different ochratoxins and their metabolites were detected. A simple procedure was also developed to confirm the presence of some ochratoxins. This involved their conversion into the methyl esters in methanol acidified with a strong acid or into the lactone opened forms in the presence of a strong base. The results showed that the newly discovered open form of OA (OP-OA) was present in significant amounts in the bile and urine of rats injected with OA or OP-OA. This form of OA, which is hightly toxic to animals, is formed in vivo and is probably involved in the overall toxicity of OA. Procedures developed in this study can be used to not only identify metabolites of OA but also can be employed to isolate and partially characterize them.

Introduction

Ochratoxin A (OA), 7-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3R-methylisocoumarin-7-L-β-phenylalanine (Fig 1 and Table 1), is a lactone containing secondary metabolite of some toxigenic species of *Aspergillus* and *Penicillium* (van der Merwe et al., 1965a, b). This toxin (OA) and some of its metabolites are found worldwide in food and feedstuffs including various crops and animal tissues and even in human blood (Steyn, 1984; Marquardt and Frohlich, 1992). OA, OC (ethyl ester of OA), and the newly discovered opened forms of OA (OP-OA, lactone opened OA) are highly toxic to animals such as pigs, poultry and dairy cattle (Marquardt and Frohlich, 1992) and to *Bacillus brevis*, eukaryotic cells (Hela) and rats (Xiao et al., 1996b). Metabolic studies of OA in the mammalian system have shown that OA is hydrolyzed to much less toxic products, ochratoxin α (Oα) and phenylalanine, by mammalian carboxypeptidase A (Doster and Sinnhuber, 1972) and by some microorganism in the gastrointestinal tract (Pitout, 1969; Madhyastha et al., 1992) and is hydroxylated to mainly 4-R-hydroxyochratoxin A (OA-OH) by the mixed function oxidases (Støren et al., 1982; Størmer et al., 1983). In addition, ochratoxin B (OB, the dechlorinated form of OA) which is about 10-fold less toxic than OA often co-occurs with OA (Marquardt and Frohlich, 1992). Pharmacokinetic and clearance studies by Li et al. (1997) demonstrated that there is a good association between the toxicity of a given ochratoxin and its half-life or a rate of clearance. Only limited studies have been carried out on the metabolites that are produced in animals other than studies with Oα and

OA-OH. Roth et al. (1988) reported that several HPLC peaks were observed in the intestine, serum, liver, bile and urine of rats injected with labelled OA. No study has reported on the nature of the degradation products of the other forms of OA (Oα, OA-OH, OB) including OP-OA. The objective of this study was to demonstrate that two simple procedures for the acetylation or the opening of the lactone ring of the ochratoxins when coupled with HPLC analysis can be used to confirm the presence of the parent compounds. In addition the fluorescent metabolites in bile and urine from rats injected with toxins were to be isolated using a HPLC gradient system that was capable of resolving more than 20 metabolites in a single run without an need for sample cleanup. The focus of this latter study was to demonstrate that the newly identified forms of OA, OP-OA-1 and OP-OA-2, were present in substancial amounts in the bile and urine of rats injected with OA or OP-OA.

Materials and Methods

Materials: Ochratoxin A and its analogs (OB; OA-OH, 4 R configuration; Oα and OP-OA) were produced or synthesized as described by Xiao et al. (1995, 1996a, b). The opened forms of the ochratoxins were opened in strong base, neutralized to pH 6.2, extracted in methanol, dried over a stream of nitrogen and stored dried until reconstituted in methanol (Xiao et al., 1996a). The methylated standards of the OA, OB, OA-OH and Oα were prepared by esterification with methanol (van der Merwe et al., 1965a, b). The polyethylene tubing was purchased from Beckon Dickinson Lab Ware, Oxnard, CA.

Sprague-Dawley rats were obtained from the University of Manitoba. Other reagents and chemicals were of analytical grade and obtained from Fisher Scientific, Winnipeg MB or Sigma Chemical Co., St. Louis, Mo. USA. All experiments were replicated a minimum of two times.

Experiment I: The objective of this study was to establish the elution patterns of the different forms of ochratoxins in an methanol-aqueous solution at different pH values. The standards contained pure forms of the different ochratoxins (OA, OP-OA, Oα, OA-OH, and OB) and each was diluted with methanol to a final concentration of 0.125 μg/ml. Samples in Figure 11 designated H_2O, acid and base contained 25 μl of the different toxins in methanol (2.5 μg toxin/ml methanol); 25 μl of H_2O, 6N HCl or 10% NaOH; and 150 μl of methanol. The mixtures were incubated at 25°C for 6 h and then analyzed by HPLC.

Experiment II: One of the objectives of this study was to determine the metabolite profiles of the ochratoxins in the bile and urine from rats that were collected from 1 to 7 h after a single intravenous injection of each of the different toxins (OA, OP-OA, Oα, OA-OH and OB). A stock solution of each toxin for injection was prepared with 1 mg of toxin in 1 ml of ethanol. One hundred microliters of stock solution (1 μg/μl ethanol) was diluted with 900 μl of saline for injection. Healthy female Sprague-Dawley rats weighing approximately 300 g were used for this study. One hundred micrograms of toxin in 1 ml of saline was administered via jugular catheter. Surgical procedures were preformed as described by Li et al. (1997). Bile and urine were collected through a bile duct cannula and a ureter catheter

at hourly intervals. Collection times were from 0 to 1 h for Oα, OA-OH and OB as they have relatively short half-lives and from 1 to 7 h for OA and OP-OA as they have relatively long half-lives (Li et al. 1997). A simple method for extraction of ochratoxins and their metabolites as described by Li et al. (1997) was used. In this procedure, bile or urine samples (100 μl) were mixed with 400 μl methanol, the mixture was centrifuged at 13,000xg for 10 min and the supernatant (20 to 50 μl) was injected onto a C-18 reversed-phase column for HPLC analysis. The rats were cared for following the guidelines of the Canadian Council Animal Care, Ottawa, ON.

The second objective of experiment II was to determine if the lactone opened forms of the ochratoxins were present in bile and urine when rats were injected with either saline (control), OA, OP-OA, Oα, OA-OH and OB and to confirm their presence by demonstrating that the two forms can be interconverted in the presence of strong acid and base. The bile and urine samples were prepared in methanol as outlined above. The supernatant samples (100 μl) containing 80% methanol (v/v) were mixed with 25 μl of water (control), 6N HCl or 10% NaOH and 75 μl of methanol. After incubated at 25°C for 6-12 h, they were subjected to HPLC analysis using a C-18 reversed-phase column.

HPLC and MS analysis: Bile and urine samples were analyzed by HPLC using the procedure described by Xiao et al. (1995), Frohlich et al. (1997) and Li et al. (1997). The HPLC system included a Waters 712 WISP sample autoinjector (Waters, Milford, MA), a LKB 2152 HPLC controller (LKB, Uppsala), a LKB 2155 HPLC column oven system. The

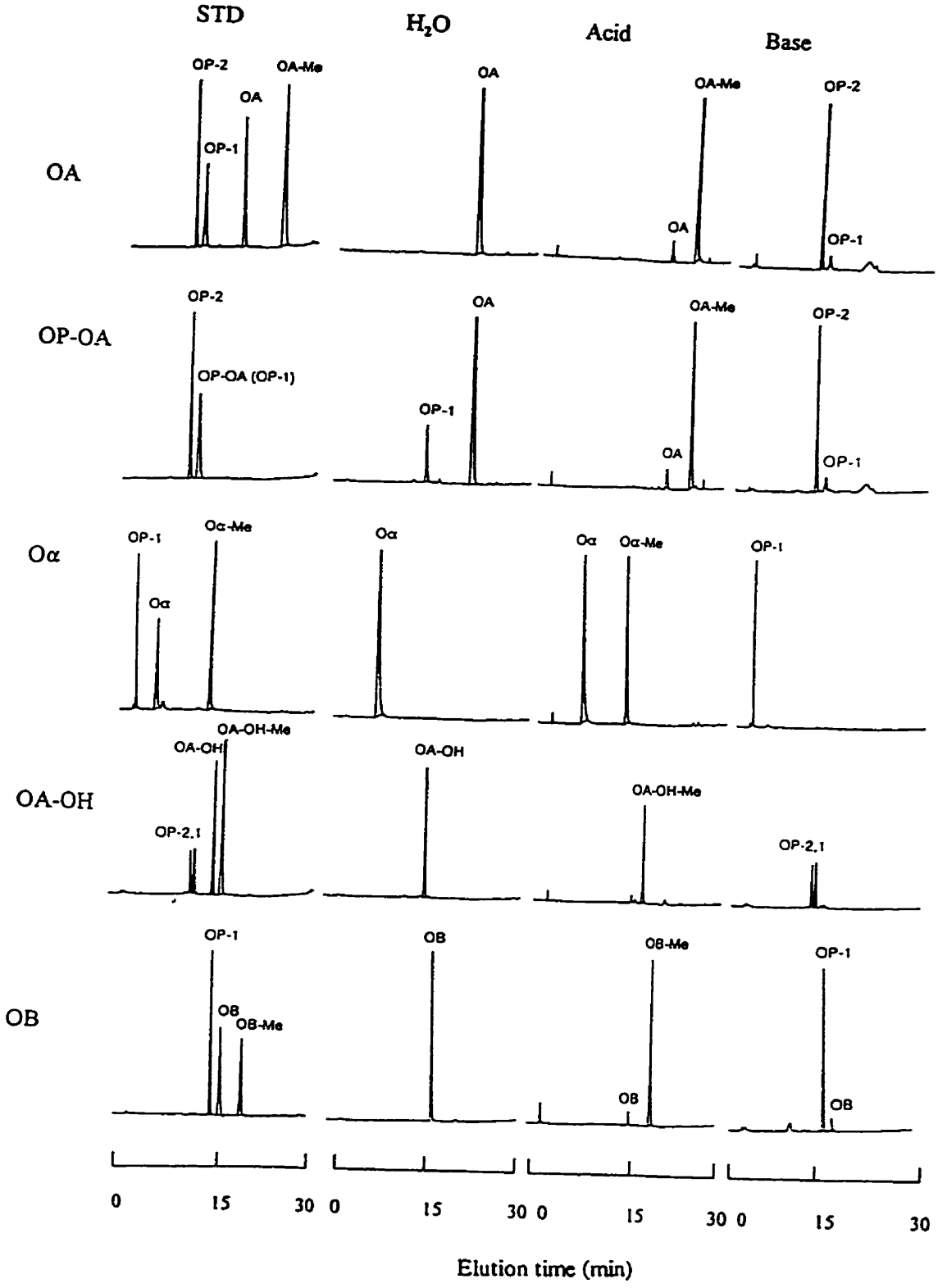
mobile phase was a gradient consisting of solvents A (methanol:isopropanol, 9:1) and B (distilled water acidified to pH 2.5 with H_3PO_4) set at a flow rate of 1.4 ml/min at 40°C. The system was programmed to deliver an isocratic mixture containing 28% of B from 0 to 5 min, 28 to 45% of B at an incremental increase of 2.4%/min (5-12 min), 45% of B for 6 min, 45 to 75% of B at an incremental increase of 3%/min (12 to 22 min), 90% of B for 8 min for washing of the column and then equilibration with 28% of B and 72% of A for 8 min prior to the next analysis. The compounds were detected using a Shimadzu (Kyoto, Japan) RF-535 fluorescence detector (excitation, 333 nm; emission, 450 nm) and recorded using a Shimadzu CR501 integrator.

The methylated toxins for NMR analyses were prepared following the general procedure outlined in Experiment II but with several modifications. In this procedure, 18 ml of each toxin including OA, OB, OP-OA or Oα (1 mg/ml methanol) was mixed with 2 ml of 6N HCl and incubated for 24 h at 25°C. The ochratoxins were extracted with the analytical grade $CHCl_3$ and dried under a stream of N_2. The El-MS of the different compounds were obtained using a 7070 HF organic mass spectrometry (VG Analytical, Manchester, England) in the Department of Chemistry, University of Manitoba.

Results and Discussions

Interconversion of the ochratoxins into different forms: Data in Fig 10 shows the HPLC elution patterns of standard forms of different ochratoxins and those that were formed

Figure 10. Conversion of ochratoxins into the methyl esters and opened forms in strong acid and base in the presence of methanol. The elution times in min and relative peak areas for the different ochratoxins are given in Table 4. The standard solutions were prepared from pure standards and diluted in methanol. The other solutions designated H_2O, acid and base were prepared as outlined in Materials and Methods, Experiment I. The names of the different compounds are: OA, ochratoxin A; OA-Me, methyl ester of OA; OP-1 or OP-2, opened 1 or 2 forms of the ochratoxins; Oα, ochratoxin α; Oα-Me, methyl ester of Oα; OA-OH, hydroxyochratoxin A; OA-OH-Me, methyl ester of OA-OH; OB, ochratoxin B; OB-Me, methyl ester of OB.


STD
H2O
Acid
Base
OA
OP-OA
Oα
OA-OH
OB
OP-2
OA-Me
OA
OP-1
OP-OA (OP-1)
Oα-Me
OA-OH-Me
OA-OH
OP-2.1
OB
OB-Me
0
15
30
Elution time (min)

in the presence of water, acid and methanol, or base and methanol. The percent methanol in the latter three samples was high (87.5% v/v) while the balance of the solution was either water, strong acid (6 N HCl) or strong base (10% NaOH). The percent conversion of the different ochratoxins after 6 h incubation at 25°C into their methyl esters in acidified methanol or into the lactone opened forms (OP-1 and OP-2) in strong alcoholic base was more than 90% for OA, OB and OA-OH (Table 4). Under similar conditions, only 40% of Oα was converted into methyl ester while its conversion into the lactone opened form was 100%. The conversion of all compounds into their methyl esters or the opened forms after 24 h of incubation at 25°C approached 100%. The exception was Oα in which the formation of the methyl ester was increased from 40% after 6 h to 70% after 24 h. Also the rate of formation of the methyl esters or the lactone opened ochratoxins increased with increasing incubation temperature (data not shown). In addition the yield of the methyl ester was influenced by the ratio of methanol and water in the mixture as a high concentration of acidified water favors hydrolysis of the ester while a high concentration of acidified methanol favors ester formation. Very little of the methyl esters (< 3%) and none of the opened forms of the ochratoxins were detected in the methanol-water preparations that had a neutral pH.

HPLC analysis of the opened form of OA (OP-OA), in contrast to the results obtained with other forms of the ochratoxin, yielded 87% OA and only 13% OP-OA when incubated for 6 h at 25 °C in 12.5% distilled water but not degassed water. Under these conditions the pH of the lactone carbonyl group of OA only occurs in the presence of a strong base such

Table 4. Conversion of ochratoxins into different products after incubation with water, HCl and NaOH in methanol for 6 h at 25°C (Experiment I) [1]

Parent toxin	Treatment	Eluted compound relative peak area (%)			
		Parent toxin	Products		
		(elution time, min)	(elution time, min)		
Ochratoxin A (OA)		OA (21.2)	OA-Me (24.1)	OP-OA-1 (14.4)	OP-OA-2 (12.8)
	H_2O	100	0 [2]	0	0
	6N HCl	5	95	0	0
	10% NaOH	0	0	6	90
Opened form of OA (OP-OA) [3]		OP-OA-1 (14.4)	OA (21.2)	OA-Me (24.1)	OA-OP-2 (12.8)
	H_2O	13	87	0	0
	6N HCl	0	5	95	0
	10% NaOH	6	0	0	94
Ochratoxin α (Oα)		Oα (7.2)	Oα-Me (13.8)	Oα-OP-1	Oα-OP-2 (2.5)
	H_2O	100	0	0	0
	6N HCl	60	40	0	0
	10% NaOH	0	0	0	100
Hydroxyochratoxin A (OA-OH)		OA-OH (15.6)	OAOH-Me (17.3)	OAOH-OP-1 (13.4)	OAOH-OP-2 (12.8)
	H_2O	100	0 [2]	0	0
	6N HCl	4	96	0	0
	10% NaOH	0	0	50	50
Ochratoxin B (OB)		OB (16.2)	OB-Me (19.6)	OB-OP-1	OB-OP-2 (15.0)
	H_2O	100	0	0	0
	6N HCl	4	96	0	0
	10% NaOH	9	0	0	91

[1] See Materials and Methods and Fig 1 for method of preparing different compounds;
[2] 0, not detected;
[3] OP-OA has same elution time as OP-OA-1. OP-OA-2 is a second form of the opened ochratoxin.

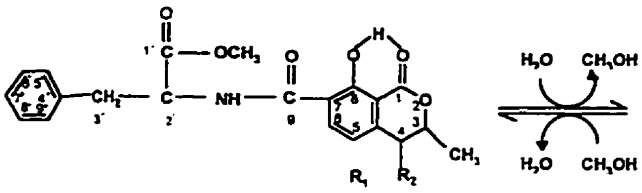
as NaOH water was less than 5.5, a pH value that favors closure (dehydration) of the lactone opened form of OA (Xiao et al. 1996a). These researchers demonstrated that its rate of closure was greatly accelerated with decreases in pH below 5.0 while it was completely stable for 12 h at pH 6.2. These observations are supported by those obtained in the current study where it was shown that OP-OA was quantitatively converted into OA in the presence of a strong acid. The open form of OA, however, is relatively stable in blood and urine that have pH values near or above 6.5. In contrast to the conversion of OP-OA into OA, the hydrolysis of OA (pH > 13) but not in the presence of a weak base such as Na_2CO_3 (pH < 11) (Xiao et al. 1996a). Two forms of the lactone opened ochratoxins were also identified in the current study and are referred to as the OP-OA-1 and OP-OA-2 forms of ochratoxin (Fig 10 and 11, Table 4). As shown in Fig 10, one of the standards when extracted from a solution at pH 6.2, and stored in 100% methanol, exists as the OP-1 form. This form of OA is stable in methanol for several days at 0°C. In contrast, when the opened form of OA is extracted from a basic solution it exists in a second form referred to as the OP-2 form of OA. These forms are interconvertable when incubated in a basic or slightly acid solution (pH 6.2). In all cases the OP-1 as compared to OP-2 forms of the ochratoxins had longer HPLC elution times suggesting that the OP-2 was more hydrophobic than OP-1. This is attributed to the fact that the C-1 carbonyl group of OP-1 would have been protonated whereas this groups in OP-2 would have been the dissociated and, as result, negatively charged. Therefore, depending on the pH of the solution, the opened lactone of the different ochratoxins can coexist as two

different opened forms, OP-1 and OP-2.

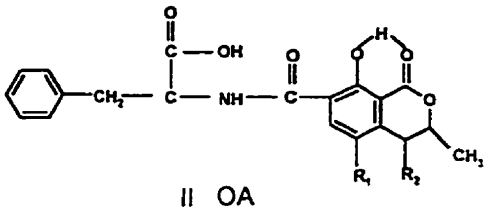
Mass spectrometric analysis demonstrated that the methyl esters of the ochratoxins that were synthesized in acidified methanol had identical masses (E1-MS) to those of the standards which were synthesized by the procedure of van der Merwe et al. (1965a, b). The *m/z* values were also the same as these reported in the literature (Cole and Cox, 1981; and Xiao et al., 1995). In addition, the methyl esters as synthesized by the two procedures in all cases had identical HPLC elution times. Methylation must have involved the 1'-hydroxyl group of OA and not the 1-hydroxyl group of OP-OA (see Fig 11) as the lactone ring would have been closed at the low pH required for the esterification reaction before a significant amount of esterification could occur.

Also the O-methyl ethers were not detected when the ochratoxins were incubated in acidified methanol as these compounds do not emit visible light when excited at 200-400 nm in contrast to their methyl esters (Xiao et al., 1995). In addition the degree of hydrolysis of the peptide bond of the different ochratoxins under the condition of incubation used in this study was less then 5% and in most cases these compounds (Oα from OA, Oβ from OB, Oα-OH from OA-OH) were not detected. Other studies (data not shown) have shown the similar results are obtained when ethanol but not propanol is substituted for methanol in the acidified-alcohol mixture. The yields of the ethyl esters are lower than those of the methyl esters while the corresponding HPLC elution times from a reversed-phase column are longer since ethanol is more hydrophobic than methanol.

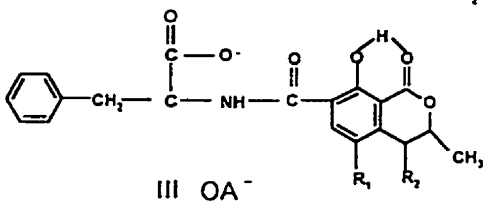
Fig 11. Proposed reaction scheme for the formation of methylated and lactone opened forms of ochratoxins with methanol in strong acid and in strong base. The different forms are: I, OA-Me, methylated OA; II, OA, protonated OA; III, OA^{1-}, single deprotonated OA; IV, OA^{2-}, double deproponated OA; V, OP-OA-1, lactone-opened OA^{2-} (double deproponated OP-OA); VI, OP-OA-2, lactone-opened OA^{3-} (triple deproponated OP-OA). The R groups for the different forms of the ochratoxins are: ochratoxin A (OA), R_1 -Cl, R_2 -H; 4-R-hydroxyochratoxin A (OA-OH), R_1 -Cl, R_2 -OH; ochratoxin B (OB), R_1 -H, R_2 -H; and ochratoxin α (Oα), R_1 -Cl, R_2-H and a hydroxyl group at carbon 9 instead of phenylalanine.



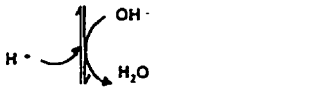
I OA-Me

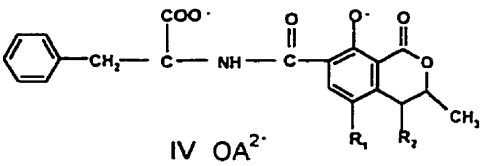


II OA

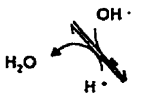


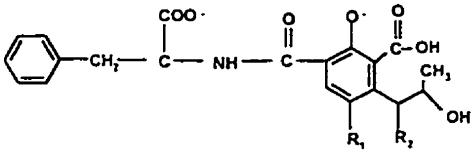
III OA^-



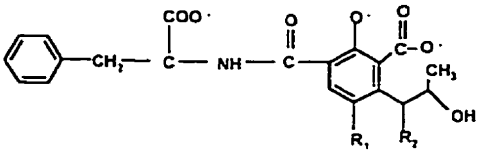


IV OA^{2-}





V OP-OA -1



VI OP-OA-2

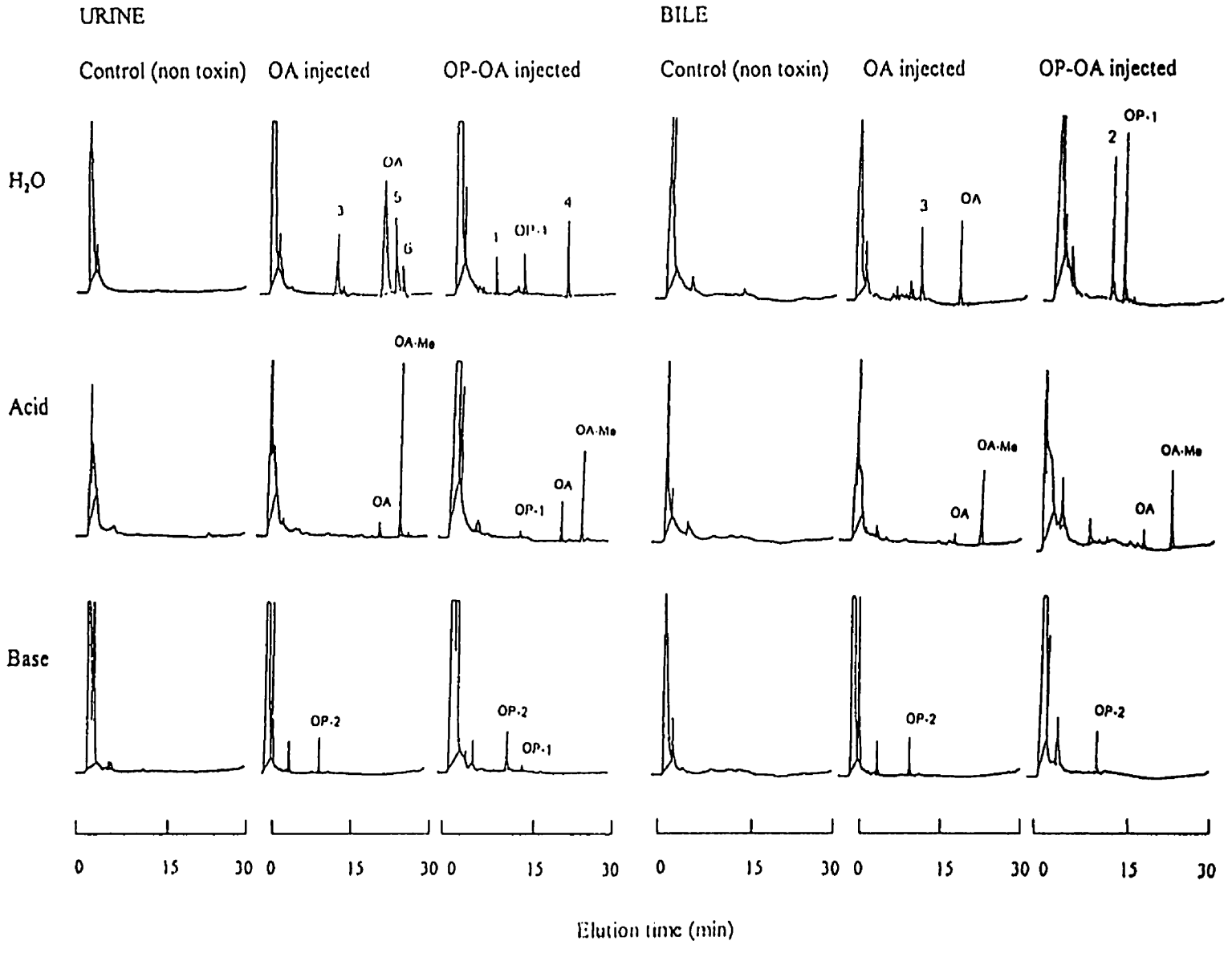
These studies demonstrated that the methyl esters, opened forms and the ring closed forms of the ochratoxins can be reversibly formed, that these forms are stable for significant periods of time if stored under the proper conditions and that they can be readily detected following HPLC analysis. In addition it was shown that lactone opened OA can exist in two forms, a protonated form (near pH 6.2) and a ionized form (basic pH) each of which has a different HPLC elution time. On the basis of the results obtained in this study and those from a former study (Xiao et al. 1996a), it is proposed that six different ochratoxins as shown in Fig 10 may occur in a solution containing water and methanol at different pH values.

An alternate procedure for preparing the methylated and the opened forms of the ochratoxins would be to extract the samples in acidified chloroform and dry the chloroform extract over nitrogen which is similar to the extraction procedure of Hult et al. (1979). The dried samples can then be diluted with methanol, aliquoted into three factions and treated with water, acid or base as outlined in Materials and Methods. This procedure compared to that used in the current study yields an extract with fewer fluorescent impurities and permits the use of an increased concentration of methanol without diluting the concentration of the analyte (data not shown). The disadvantages of the procedure are (a) the more hydrophilic metabolites may have limited solubility in acidified chloroform compared to that in methanol; (b) the extraction procedure is much more involved than that used in the current study; (c) the improvements in the quality of the chromatograms were not dramatic (data not show); (d) possible to identify the presence of the OP-OA in tissue extracts.

Metabolites of the different ochratoxins: The HPLC pattern of the bile and urine of rats injected with OA and OP-OA (Fig 12) and Oα, OB and OA-OH (Fig 13) demonstrated the presence of not only the parent compound but also several metabolites, particularly in the bile. A typical HPLC elution pattern for control rats (saline treatment only) demonstrated that there essentially were no fluorescent compounds in urine and bile that coeluted with the predominate fluorescent compounds in the bile and urine from the ochratoxin treated rats. The total number of metabolites in addition to the parent compounds that were detected in the bile and urine when OA and OP-OA, Oα, OA-OH or OB were injected into rats were 6, 4, 4 and 2, respectively. Many other metabolites in addition to those shown in Fig 12 and 13 were readily and consistently visible in the chromatograms when larger quantitatives of the samples were injected. Roth et al. (1988) also reported that there were many metabolites in bile. These data therefore suggested that a considerable number of different metabolites of the different ochratoxins are present in bile and urine, especially in the bile and especially those formed from partially metabolized compounds such as Oα, OA-OH, OB, and OP-OA. Collectively more than 20 different metabolites of OA were identified, each with different HPLC elution times.

The current research, however, has focussed on some of the main metabolites and not those that were present in low concentrations. An overall summary of the predominate peaks that were detected, their relative concentration as estimated from peak areas and their elution times is given in Table 5. All of the samples, in addition to being diluted with methanol, were

Fig 12. HPLC profile of ochratoxins in bile and urine from rats injected with carrier (saline), OA or OP-OA. The samples (100 μl) were diluted with 25 μl each of either water, 6N HCl or 10% NaOH and 75 μl methanol, incubated for 6 to 12 h at 25°C and analyzed by HPLC. See Materials and Methods, Experiment II for further detail.


URINE
BILE
Control (non toxin)
OA injected
OP-OA injected
Control (non toxin)
OA injected
OP-OA injected
H2O
Acid
Base
OA
OA-Me
OP-1
OP-2
0
15
30
Elution time (min)

incubated in the presence of acidified methanol or methanol in strong base (Fig 12 and 13, Table 5). These two latter treatments provided further evidence on the identity of the different compounds. The studies in which OA was injected into the rat demonstrated that two major peaks were identified in both the urine and the bile, one of which had an elution time identical to that of OA and the other which had an elution time that was the same as that of OP-OA (peak 3), the lactone opened form of OA. In addition urine contained two other major peaks (5 and 6) while bile appeared to contain several other minor peaks prior to that of peak 3. When the bile and urine samples were incubated in acidified methanol all of the OP-OA and most of OA disappeared with a new peak appearing that had an elution time that was the same as OA-Me. Likewise, when the two samples were incubated in a basic solution, the OA peak disappeared and the peak that had the same elution time as OP-OA increased in size. Injection of OP-OA into the rats instead of OA yielded chromatographic patterns similar to but not identical to those obtained when OA was injected. The results show that it was converted in the animal into OA. These data provide good evidence that two of the major HPLC peaks in the aqueous extracts from bile and urine were OA and OP-OA and that considerable amounts of OP-OA are present in both the bile and the urine of rats injected with either OA or OP-OA.

Studies with Oα, OA-OH and OB demonstrated that the major HPLC peaks in the bile and urine of rats injected with these compounds were the parent compounds with smaller amounts of several other metabolites being detected (Fig 13, Table 5). As indicated above, a total of 20 new fluorescent peaks were identified in the different chromatograms. They

Fig 13. Confirmation of OA and its major metabolites including Oα, OA-OH and OB in bile and urine from treated rats by conversion tem into the methyl esters and opened forms.

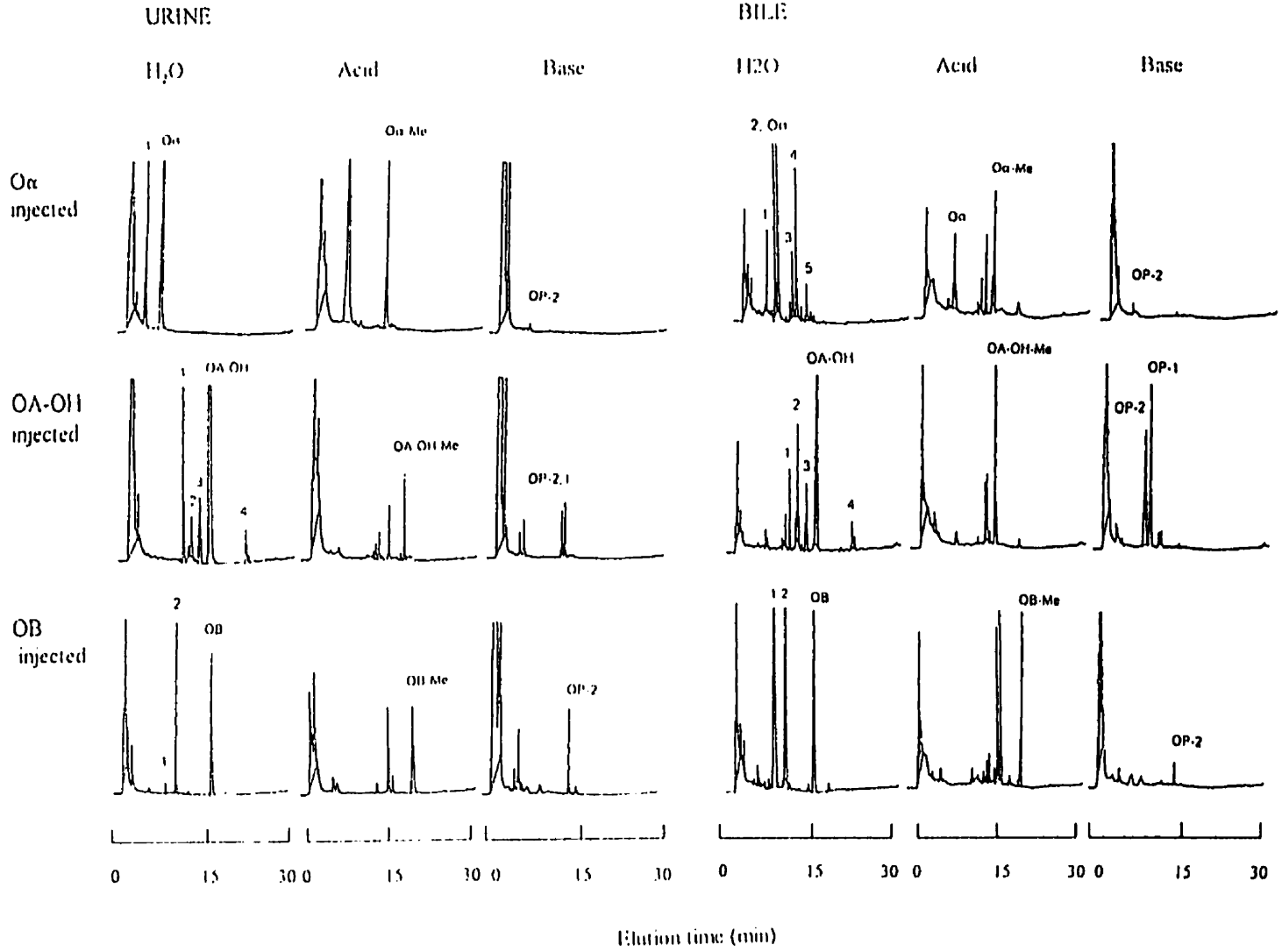

URINE
BILE
H2O
Acid
Base
H2O
Acid
Base
Oα injected
OA-OH injected
OB injected
Oα
Oα Me
OA OH
OA OH Me
OB
OB Me
OP-2
OP-2.1
OA-OH
OA-OH-Me
OP-1
0
15
30
Elution time (min)

Table 5. Percent relative peak areas following HPLC analysis for parent toxins and their metabolites in bile and urine following a single i.v. injection into rats (Experiment 2)

Injected compound [1]	Elution time (min)	Relative peak area of eluted compound (%)	
		Urine	Bile
Open OA (OP-OA)	8.1 (1)[2]	21	0
	11.2 (2)	0	46
	14.2 (3, OP-OA-1)[3]	26	54
	21.2 (4, OA)	52	0
Ochratoxin A (OA)	14.2 (3. OP-OA-1)	17	40
	21.6 (4, OA)[3]	34	60
	24.6 (5)	24	0
	26.4 (6)	+[4]	0
Ochratoxin α (Oα)	4.3 (1)	30	13
	6.7 (2)	0	27
	7.2 (Oα)[3]	70	27
	9.5 (3)	0	12
	10.0 (4)	0	16
	11.6 (5)	0	+
4-Hydroxyochratoxin A (OA-OH)	11.1 (1)	21	13
	12.5 (2)	+	19
	14.0 (3)	+	11
	15.6 (OA-OH)[3]	62	53
	21.7 (4)	+	+
Ochratoxin B (OB)	9.9 (1)	+	40
	12.5 (2)	54	40
	16.2 (OB)[3]	46	20

[1] Amount of each toxin injected was 100 μg/ 300 g of rat;
[2] Numbering of the metabolites as indicated in Fig 13 and 14;
[3] Indicates parent compound;
[4] +, compound was detected but its concentration was less than 10%.

presumably represent breakdown products of the parent compounds and are different from each other and those of OA since they have different elution times. In addition, numerous other minor peaks were evident most of which did not coelute with peaks in the control urine and bile samples. The addition of acid or base plus methanol to the samples resulted in the appearance of several new peaks and the disappearance of several or most of the original peaks. Acid treatment of urine and bile in the presence of methanol resulted in the disappearance of part of the Oα peak and the appearance of a new peak that had an elution time the same as that of Oα-Me. Similarly most of peaks 1 to 5 decreased in size and several other minor peaks were formed. It is not known, however, if some of these peaks were converted into Oα-Me which would suggest that they would have been a conjugate of Oα. Each of the metabolites would have to be isolated in pure form to confirm the nature of their interconversion into a new product in acidified methanol. In base nearly all of the peaks disappeared with a minor peak being formed (OP-2) which had an elution time that was the same as the lactone opened form of Oα and a major peak in both bile and urine that was eluted just after the void volume peak (Fig 13). The effects of the acidified methanol and base treatments of bile and urine from OA-OH and OB injected rats were generally similar to the effects shown for Oα. These results suggest that the new major peaks in bile and urine from rats injected with Oα, OA-OH and OB were probably metabolites of these compounds and that they contained an intact isocoumarin moiety since they still were fluorescent. Many of the new compounds appeared to also contain a carboxyl group other than that associated with

the C-1 carboxyl of the opened ochratoxins as it would have been converted into its non-reactive lactone at the low pH of the reaction. This study demonstrated that there are many fluorescent degradation products of OA and its metabolites in bile and urine of rats injected with OA and some of its metabolites, has confirmed the presence in bile and urine of a new form of OA (OP-OA) and has provided a basis for the isolation of each metabolite as the HPLC gradient used in this study resulted in the separation of many different forms of the ochratoxins (more than 20) present naturally in urine or bile without a need for an elaborate sample cleanup procedure. The number of different metabolites of OA that were separated included OP-OA, Oα, OA-OH, OB; when OA or OP-OA were injected; and 6, 5, 3 and 2, respectively, when Oα, OA-OH and OB were injected. In addition several other minor breakdown products were detected. In all cases the different peaks remained sharp throughout the gradient with high peak to width ratios. Preliminary studies have demonstrated that the HPLC gradient and size of column can be further modified to facilitate collection of large quantities of each metabolite in pure form for further identification. The ability to demonstrate that all of the different forms of the ochratoxins can be readily and reversibly interconverted into their methyl esters or lactone-opened form also provides a means of confirming the identity of the parent compounds as it is unlikely that coeluting contaminates would behave identically when treated in a similar manner. It was also shown that ethanol can replace methanol for esterification of the ochratoxins to form the ethyl esters of the different compounds (data not shown). These ethyl esters have longer HPLC elution times than the

methyl esters and therefore provide a further means of confirming the presence of a particular ochratoxin. As indicated above this study confirmed the observations of Xiao et al. (1996a) that a new form of OA, OP-OA, was present in the bile and urine of rats injected intravenously with either OA or OP-OA and that these fluids contained a significant amount of OP-OA. The relative toxicity of OP-OA and its possible mode of action was not established. Nevertheless it was shown to be highly toxic when injected intravenously but not when injected intraperitoneally into mice (Xiao et al. 1996a). Research in our laboratory also demonstrated that the survival time of rats injected intravenously with OP-OA was much shorter than that obtained when a similar concentration of OA was injected (unpublished data). This suggests that the lactone opened form of OA is more toxic than OA. Further research is required to identify metabolites and metabolic pathways for the degradation of OA and to establish the importance and role of OP-OA in the overall toxicity of OA.

MANUSCRIPT III

Pharmacokinetics of Ochratoxin A and its Metabolites in Rats

Li, S.[1], R. R. Marquardt [1], A. A. Frohlich [1], T. G. Vitti and G. Crow[1]

Department of Animal Science[1] and Faculty of Pharmacy[2],

The University of Manitoba

Abstract

Ochratoxin A (OA) is a mycotoxin that is produced on moist grain. It is commonly found in the blood of swine in western Canada and is a potent nephrotoxic, carcinogen and immunosuppressive agent. The pharmacokinetic characteristics of six analogs of OA including OA, OB (OA without chloride), OC (OA ethyl ester) and some metabolites, such as Oα (OA without phenylalanine), OA-OH (hydroxylated OA) and a newly discovered form of OA, OP-OA (lactone opened ring of OA), were investigated in rats after a single intravenous administration of the compounds. All of the ochratoxin analogs were distributed following a two compartment open model. The elimination half-lives of OA, OP-OA, Oα, OA-OH, OB and OC were 103 ± 16, 50.5 ± 2.8, 9.6 ± 2.3, 6 ± 0.9, 4.2 ± 1.2 and 0.6 ± 0.2 h, respectively. Total body clearance of OA, OP-OA, Oα, OA-OH and OB via the bile, urine and metabolic routes were 3.1, 3.6, 40, 65 and 43 ml/h kg, respectively. OA, OB and Oα were mainly cleared in the urine (≥48%), OA-OH in the bile (41%) and OP-OA as metabolites (43%). Metabolism accounted for 43, 44, 33 and 29% of the total clearance of OA, Oα, OA-OH and OB, respectively. It is concluded that OA has a long half-life and is very slowly cleared from the body and that its metabolites are cleared at a much faster rate with much shorter half-lives. Procedures should be devised to enhance the conversion in the body of OA to Oα, OA-OH or other metabolites as this would shorten its half-life and therefore its toxicity.

Introduction

The ochratoxins are a family of mycotoxins which have structurally related derivatives. Included are ochratoxin A (OA), B (OB), C (OC), 4-hydroxyochratoxin A (OA-OH), ochratoxin α (Oα), ochratoxin β (Oβ) and the open form of ochratoxin A (OP-OA) (van der Merwe et al., 1965a,b; Steyn and Holzapfel, 1967a; Steyn, 1971, 1984; Hutchinson et al., 1971; Xiao et al., 1995, 1996a). These naturally occurring mycotoxins are found worldwide in food and feedstuffs including various crops, animal tissues and even in human blood (Steyn, 1984). OA, OC and the less toxic dechloro analog of OA, OB, are produced by *Penicillium* and *Aspergillus* species of fungi (de Scott, 1965). OA and OC are extremely toxic to animals, such as pigs, poultry (Steyn, 1984; Chang et al., 1979) and dairy cattle (Wei et al., 1973) The ochratoxins are potent carcinogens, hepatotoxins and nephrotoxins and have been implicated in some human diseases including an irreversible and fatal kidney disease referred to as Balkan endemic nephropathy. The metabolites, OA-OH and Oα, have been found in urine and feces (Hult et al., 1979; Kiessling et al., 1984; Chang and Chu, 1977). OA and OC have a similar structure and also similar toxicity. OB which has a H-group instead of a Cl-group is approximately 10-fold less toxic than OA while the metabolites of OA, Oα and OA-OH, are not toxic (Marquardt and Frohlich, 1992). The new form of OA, OP-OA, has been shown to be highly toxic when injected intravenously (Xiao et al.,1996a). Some pharmacokinetic studies with OA have been carried out on several different species of animals. The results have shown that the pharmacokinetic profile of OA follows a two

compartment model with variation among different animals (Galtier et al., 1979; Galtier et al., 1981; Chang and Chu, 1977). Limited studies with OB in rats indicate that there is a large difference in the pharmacokinetic profiles for OA and OB (Hagelberg et al., 1989). There have been, however, no reports on the pharmacokinetics for OC, OA-OH, Oα, OP-OA and on the clearance of the ochratoxins. The objective of this study was to obtain more comprehensive information on the disposition of OA and its metabolites in rats following single intravenous administration of the different ochratoxins as determined from their half-life values and extent of clearance via the kidney, the bile and metabolism.

Materials and Methods

Sources of ochratoxin, other metabolites of OA and rats. Six ochratoxin-related compounds including OA, Oα, OA-OH (4R configuration), OB, OC and OP-OA were used. OA, Oα, OA-OH, OB, and OP-OA were produced or synthesized according to the methods of Xiao et al. (1995). OC was prepared by esterifying OA with ethanol (Fuchs et al., 1984). The structures for each compound are shown in Fig 1 and Table 1. The polyethylene tubing was purchased from Beckon Dickinson Lab Ware, Oxnard, CA. Other reagents and chemicals were of analytical grade and obtained from Fisher Scientific, Winnipeg, MB or Sigma Chemical Co., St. Louis, MO, USA. Healthy adult female Sprague-Dawley rats weighing approximately 300 g (270-350 g) were obtained from the University of Manitoba animal colony.

Experimental design. Three different experiments were performed. They included the determination of (1) the half-life of OA and its analogs, (2) the clearance (total, renal and biliary) of these compounds from the body, and (3) the half-life of OA in the tissue of rats. In the first experiment, 100 μg of OA, OP-OA, Oα, OA-OH, OB or OC in 1 ml saline were injected intravenously. Each toxin was dissolved in ethanol (1 mg/ ml), and 100 μl of the solubilized toxin was mixed with 900 μl saline before injection into the rats via a jugular catheter. Slightly more than 100 μl of blood was obtained for each sampling time from the carotid artery when the half-life of the toxin was less than 10 h (Oα, OA-OH, OB and OC), and for OA and OP-OA during the first day of the experiments. In these studies, the injected animals were first anesthetized and tubes were inserted into the trachea and the carotid artery immediately before initiation of the experiment. Blood for toxins that had a long half-life (OA and OP-OA) was collected from the orbital sinus of the rats on the second and subsequent days as indicated in Figures 2 and 3. At least 6 samples were collected from each rat during the distribution phase and the elimination phase. The number of rats for each toxin was either 6 (OA or OP-OA), 3 (Oα, OA-OH, OB and OC). Blood was analyzed for the specific toxins as indicated subsequently.

The clearance experiments involved collection of blood, bile and urine samples. A requirement of the model is that all samples be collected simultaneously, therefore, each bile and urine samples were collected for a known period of time (usually of 1 h duration) and a blood sample was obtained at the mid-point time of the collections. Blood, urine and bile

were obtained from the same animal over the entire time course of the study for those toxins that had a short half-life. The times for collection after administration of toxin for bile and urine were at hourly intervals from 2 to 5 h , and from 7 to 10 h for Oα (n=6 rats), OA-OH (n=6 rats) and OB (n=6 rats) while blood was collected at hourly time intervals including 2.5, 3.5, 4.5, 7.5, 8.5, 9.5 h. The collection times for bile and urine for OP-OA and OA of treated rats were from 2 to 10, 27 to 30 and 72 to 75 h at hourly time intervals. Blood was collected at the mid-point of each sample collection time. The toxins that had the longer half-lives (OA and OP-OA) required a new set of animals for each time period. New animals were required for each time period as they did not recover sufficiently between time periods to be reused. In these studies a set of 9 rats were injected with each toxin and 3 rats were used for the first time periods (from 2 to 10 h) and 3 rats for the second time period (27 to 30 h) and another 3 rats for last time period (72 to 75 h). The other procedures were the same as described for the shorter studies.

Preliminary studies demonstrated that it was not desirable to collect urine from the cannulated bladder as it always contained a variable volume of urine which would have confounded the concentration data. This was avoided by collection of urine directly from the ureter of rats that had one kidney removed to ensure that all of the urine was voided through the remaining kidney (Smyth and Penner, 1995). It is not known, however, if the hydrodynamics of the one kidney model is identical to that of the two kidney model. A seven day adjustment period prior to the experiment allows sufficient time for hypertrophy of the

single kidney (Smyth, D., Personal Communication). HPLC (high performance liquid chromatography) analysis of samples and statistical analysis of the data were as given subsequently.

The final experiment established the rate of conversion of OC to OA and the half-life of OA in the tissue when either OA or OC was administered. The first part of the study was carried out to determine the rate of disappearance of OC and the rate of appearance of OA in the blood of rats injected within OC. The procedures were as outlined for the initial experiment. Four rats were injected with OC and blood was collected from each rat at the time intervals indicated in Figure 16. Blood was analyzed for OC and OA. In the second part of the study, OA or OC was administered i.v. to 32 rats, 16 for each toxin. Four rats were used for each time period for each toxin. Rats were sacrificed at 2, 24, 48 and 96 h after toxin administration, and blood, liver, kidney, muscle and heart were immediately removed, blotted, weighted and extracted for OC and OA analysis.

Animal treatment and surgical procedures for half-life studies of toxin in blood and for the clearance studies. Tracheotomy was performed using a polyethylene tubing (i.d. 1.67 mm, o.d. 2.42 mm) after the rats were anaesthetized with Nembutal (i.p. 450 mg/kg). The carotid artery was cannulated with a polyethylene tubing (i.d. 0.58 mm, o.d. 0.965 mm) to obtain arterial blood samples while the jugular vein was catheterized with a similar type tubing (i.d. 1.14 mm, o.d. 1.57 mm) for the continuous infusion of heparin in saline (flow rate of 500 μl/min) and for the administration of the ochratoxins. Heparin and saline were

administered with the aid of a model 2720 infusion pump (Harvard Apparatus, South Natick, MA 01760, USA). A bile duct cannula using similar tubing to that described above (i.d. 0.28 mm, o.d. 0.61 mm) provided a steady flow of the bile throughout the collection period. Urine samples were obtained from a polyethylene ureter catheter (i.d 0.58 mm, o.d. 0.965 mm) in rats that had one kidney removed at least one week prior to the experiment. Bile and urine were collected at hourly intervals. The rats remained anesthesized during the entire course of the experiment. Supplemental heat for the rats was provided by means of a thermostated heating pad. The overall procedure was similar to that outlined by Mulder et al. (1981). The experiments were carried out according to the guidelines of the Canadian Council of the Animal Care, Ottawa, ON, Canada.

Dosing, sample collection, extraction and analysis. Toxin (100 μg) was administered as a single i.v. dose to each rat via the jugular catheter. Blood samples of slightly more than 100μl were obtained from the arterial catheter at various time intervals after iv injection of the toxins. The samples were extracted using the procedure described by Hult et al. (1979). Briefly, 100μl of the whole blood was transferred into 1.5 ml of distilled water followed by the addition of 1.5 ml of saline (0.145M NaCl). The sample was mixed well, 10 ml of 0.05N HCl-0.1M $MgCl_2$ and 6 ml of $CHCl_3$ were added, and the mixture was shaken for 10 min in a mechanical shaker. The sample was placed in an ice bath for 10 min and centrifuged for 15 min at 1300xg. Four millilitres of the chloroform layer were removed with the aid of a syringe that had an attached cannula and the contents were transferred into a glass vial. Deionized

water (1.5 ml) was added , the sample was throughly mixed and the mixture was centrifuged at 1300xg for 10 min. Part of the $CHCl_3$ layer (3 ml) was transferred into an opticlear vial and evaporated using a N_2 stream. The dried samples were stored at -20°C. Samples prior to HPLC analysis were reconstituted with 500-1000 μl of methanol, sonicated for 1-2 min and 200 μl were added to HPLC vials for analysis. The open form of OA was extracted directly into methanol. In this procedure, 100 μl of blood was mixed with 400 μl of methanol. The samples were centrifuged at 13,000xg for 10 min and the supernatants were used for HPLC analysis.

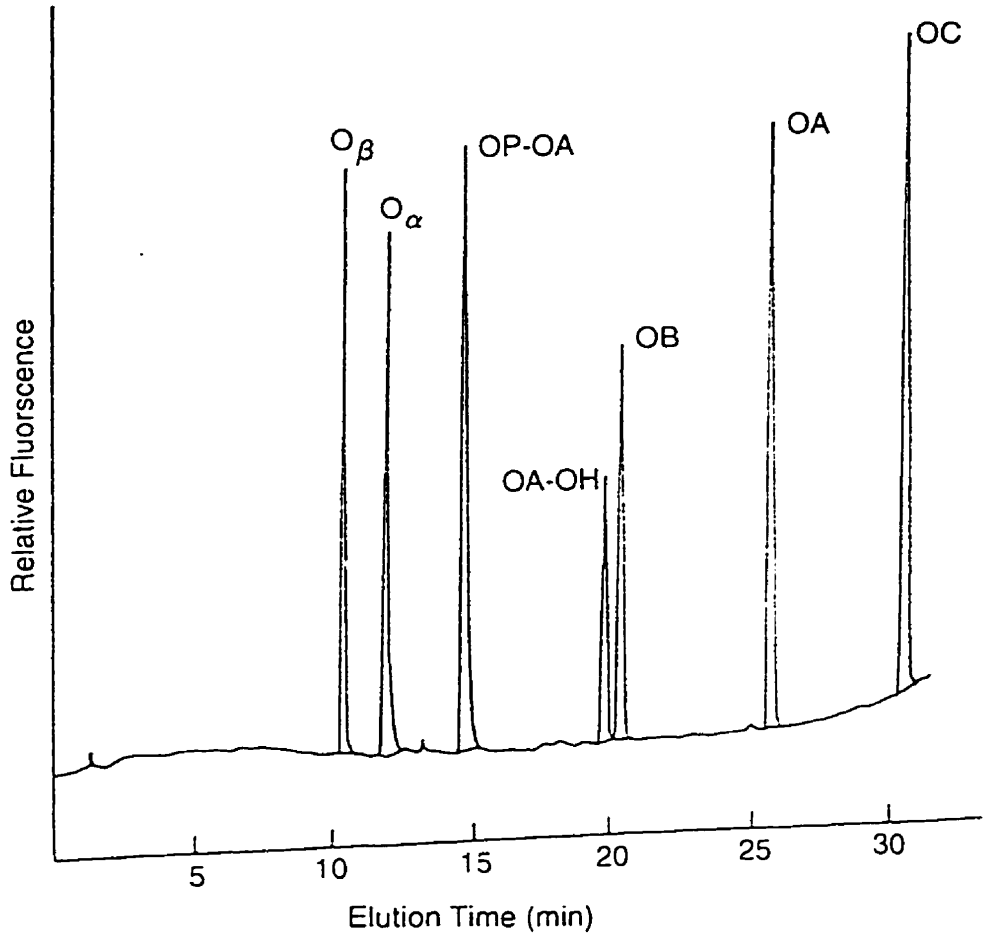
Bile and urine samples were obtained from the bile and ureter catheters. Urine (100 μl) in a 1.5 ml Eppendorf vial was mixed with 0.5 ml of precipitating solution that consisted of 0.2 g of EDTA, 1.67 g of metaphosphoric acid and 30 g of sodium chloride in 100 ml of double distilled water and 1 ml of methanol. Bile was diluted 5-fold with 400 μl of methanol. Both urine and bile mixtures were centrifuged at 1,300 xg for 10 min and the supernatant was injected onto an HPLC column.

Tissue samples from the last experiment were extracted using the following procedure. One gram of tissue was homogenated with 10 ml of 0.1M $NaHCO_3$, the pH was adjusted to 3 with concentrated H_3PO_4, and 12 ml $CHCl_3$ was added. The mixture was homogenized (Janke and Kunkel, Ultr-Turra T25, homogenizer) for 2 min, shaken for 20 min in a rotary shaker and centrifuged at 3000xg for 15 min. Four millilitres of $CHCl_3$ containing the toxin was transferred into a 10 ml vial and the sample was dried under a stream of nitrogen. It was

reconstituted with 0.5 ml of 80% methanol and sonicated before injection onto the HPLC column.

HPLC analysis. Blood, bile, urine and tissue samples were analyzed by HPLC, using a procedure similar to that described in our laboratory (Frohlich, et al., 1988; Xiao et al., 1996a,b). The HPLC system included a Waters 712 WISP sample auto-injector (Waters, Milford, MA), a LKB 2152 HPLC controller (LKB, Uppsala, Sweden), LKB 2150 HPLC pump, a LKB 2155 HPLC column oven system; and a RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) set at an excitation of 330 nm and an emission of 450 nm. The mobile phase was a gradient consisting of water acidified to pH 2.1 with H_3PO_4 (A) and 90% methanol with 10% isopropanol (B) set at a flow rate of 1.5 ml/min. The system was programmed to deliver 30 to 48% B at an incremental increase of 1.8%/min (1-10 min), 48% B (10-16 min), 48 to 69% B at an incremental increase of 2.3%/min (16-25 min), 69 to 90% B at an incremental increase of 10%/min (25-28 min), 90% B (28-35 min) and 30% B (35-40 min). The elution times (min) of the ochratoxins in the standard samples were: Oβ, 9.9; Oα, 12.2; OP-OA, 14.6; OA-OH, 17.6; OB, 18.1; OA, 25.6 and OC, 31.2 min, respectively (Fig 14). Several new peaks appeared in the bile and urine of rats treated with the different ochratoxins. These peaks were not present in the bile and urine of control rats prior to administration of toxin. It was assumed that these new peaks were metabolites from OA, Oα, OB, OA-OH, OP-OA and OC and that they had absorbency and emission values that were the same as the parent compound. Accurate values are not available since standards of these

Figure 14. Reverse-phase HPLC elution profile of different mixtures the ochratoxins. The name of the compounds, the abbreviation and the elution times (min) are: ochratoxin β, Oβ, t=9.9; ochratoxin α, Oα, t=12.2; the open form of ochratoxin A, OP-OA, t=14.6; 4-hydroxyochratoxin A, OA-OH, t=17.6; ochratoxin B, OB, t=18.1; ochratoxin A, OA, t=25.6; ochratoxin C, OC, t=31.2 min. each standard was more than 99% pure (Xiao et al., 1995). See Materials and Methods for detail.


Relative Fluorscence
O_β
O_α
OP-OA
OA-OH
OB
OA
OC
5
10
15
20
25
30
Elution Time (min)

compounds have not been prepared.

Pharmacokinetic analysis. A two-compartment open model was used for the calculation of pharmacokinetic parameters. The ochratoxin concentrations in blood and tissue vs time curves were fitted to the biexponential expression (Gibaldi and Perrier, 1975; Shargel and Yu, 1985):

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

where single dose intravenous administration, where Cp is the blood concentration at time t, A and B are y intercepts, α and ß are the distribution and the terminal elimination rate constants, respectively. These constants were determined by computer using the SAS non-linear regression program data analysis system (SAS Institute, Inc., 1989). The biological half-life (t½ß) was determined from the equation: $t_{½} = 0.693/ß$. The area under the blood concentration-time curve from t=0 to t=t (AUC_{0-t}) was estimated by the trapezoidal rule (Shargel and Yu, 1985). The $AUC_{0-\infty}$ from time zero to infinity after toxin administration was obtained by the following equation:

$$AUC_{0-\infty} = A/\alpha + B/ß$$

where A, B, α and ß are constants, as indicated previously. The volume of distribution of the central compartment (Vc) and the apparent volume of the peripheral compartment (Vp) were determined by the following equations (Gibaldi and Perrier, 1975):

$$Vc = dose/(A+B) \times B.W.$$

$$Vp = (Vc \times k_{12})/k_{21}$$

$$k_{21} = (A\beta+B\alpha)/(A+B)$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10}$$

$$k_{10} = \alpha\beta/k_{21}$$

where B.W. is body weight of the rat, k_{10} is the overall elimination rate constant, k_{21} and k_{12} are distribution rate constants.

Urinary and biliary excretion and metabolic interconversions were considered in the clearance studies. Cl_R is renal clearance and Cl_B is biliary clearance. Renal clearance was obtained by the equation (Gibaldi and Perrier, 1975):

$$Cl_R = Du^{\infty}/ AUC_{0-\infty} = Du^{t^*}/AUC_{t1-t2}$$

where Du^{t^*} is amount of unchanged toxin excreted in the urine over the collection period, $t^* = t_1-t_2$, and AUC_{t1-t2} is the area under the toxin blood concentration-time curve over the collection period. Biliary clearance (Cl_B) was similarly determined:

$$Cl_B = D_B{}^{t^*}/AUC_{t1-t2}$$

where $D_B{}^{t^*}$ is the amount of unchanged toxin excreted in the bile over the collection period, and the total clearance (Cl_T) was determined by the following equation (Gibaldi and Perrier, 1975):

$$Cl_T = k_{10} \times Vc$$

where k_{10} and Vc are as defined previously. Metabolic clearance (Cl_M) of the administered toxin was calculated according to the equation (Gibaldi and Perrier, 1975):

$$Cl_M = Cl_T - Cl_R - Cl_B.$$

Results and Discussion

Pharmacokinetic Profile: Typical semi-logarithmic plots of the concentration of ochratoxin A and its metabolites in blood following single intravenous injections of the different compounds are shown in Fig 15. The concentration for each compound in the blood declined in a biexponential manner, which suggests that the distribution of all the compounds followed a two-compartment open model in a manner similar to that reported by Galtier et al. (1979) and Hagelberg et al. (1989) for OA. After computerized fitting, the following equations were obtained:

OA (n=6); $Cp = 1074.76e^{-0.2442t} + 549.3e^{-0.0067t}$

Oα (n=3); $Cp = 1272.36e^{-0.0251t} + 581.21e^{-0.00166t}$

OA-OH (n=3); $Cp = 1436.71e^{-0.0307t} + 421.47e^{-0.00176t}$

OB (n=3); $Cp = 1823.116e^{-0.0424t} + 961.64e^{-0.00232t}$

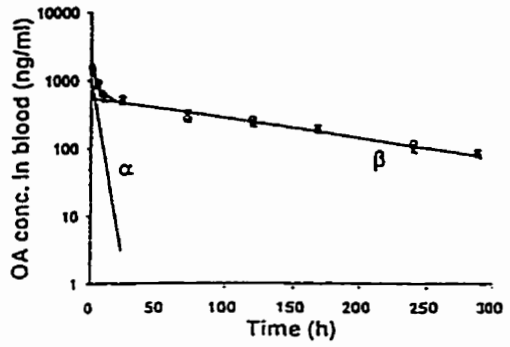
OC (n=3); $CP = 460.09e^{-0.1413t} + 150.12e^{-0.01397t}$

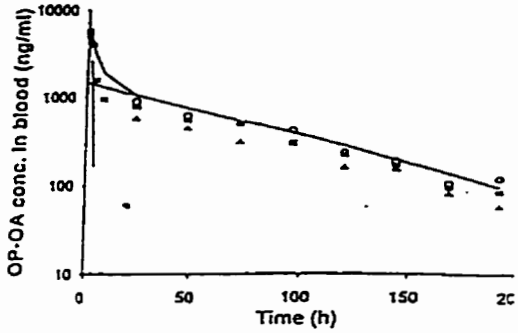
OP-OA (n=6); $CP = 4601.82e^{-0.2478t} + 1051.54e^{-0.01316t}$

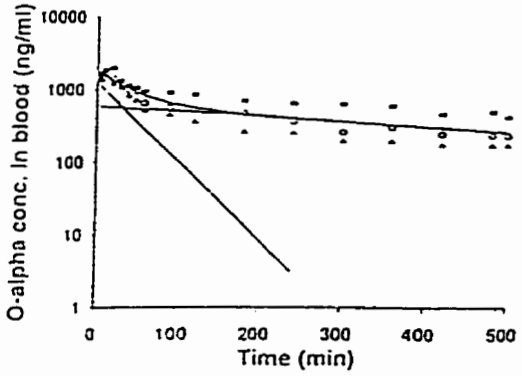
where Cp is the average toxin concentration in blood at each time (t is h for OA and OP-OA, other values are in min). A summary of the individual pharmacokinetic parameters of OA and its metabolites including t½α, t½β, Vc, Vp, $AUC_{0-\infty}$ and clearances are presented in Table 6.

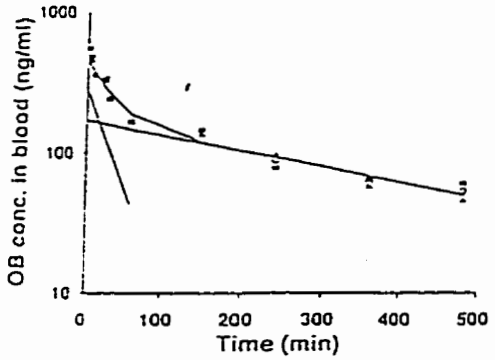
The data for the initial distribution phase (t½α, distribution half-life) show that the half-lives were relatively short. The distribution half-life (t½α) of OA from the blood of rats was 160 min (2.7 h, Table 6). This value is similar to those obtained in previous studies with

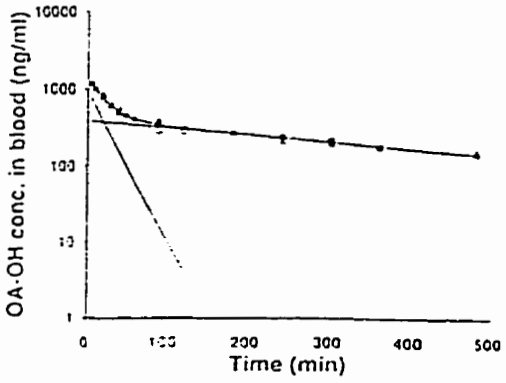
Fig 15. Semiloarithmic plots of the concentration of ochratoxin A (OA); the open-form ochratoxinA (OP-OA); ochratoxin α, Oα (Oα); 4-hydroxyochratoxin A (OA-OH); ochratoxin B (OB) and ochratoxin C (OC). α and β, the first-order rate constant for the distribution and eliminination phase, respectively, were obtained from the slope of the distribution phase (α) and elimination phase (β). They were used to calculate the corresponding half-life values given in Table 2. Blood was collected at the indicated time intervals after the toxin injection. The collection time were 0.25, 0.5, 1, 5, 8, 10, 24, 72, 120, 168, 240 and 288 h for OA; 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 and 198 h for OP-OA; 2, 5, 10, min intervals up to 60, at 30 min intervals up to 120 min, at 60 min intervals up to 480 min for Oα, OA-OH and OB; and 2, 5, 10, 20, 25, 30, 40, 50, 60, 120, 150 and 180 min for OC. The number for each of the 3 rats used for each toxin is indicated by different symbols (■, O, ▲). See Materials and Methods for further detail.


OA conc. in blood (ng/ml)
10000
1000
100
10
1
α
β
0
50
100
150
200
250
300
Time (h)


OP-OA conc. in blood (ng/ml)
10000
1000
100
10
0
50
100
150
Time (h)


O-alpha conc. in blood (ng/ml)
10000
1000
100
10
1
0
100
200
300
400
500
Time (min)


OB conc. in blood (ng/ml)
1000
100
10
0
100
200
300
400
500
Time (min)


OA-OH conc. in blood (ng/ml)
10000
1000
100
1
0
200
300
400
500
Time (min)

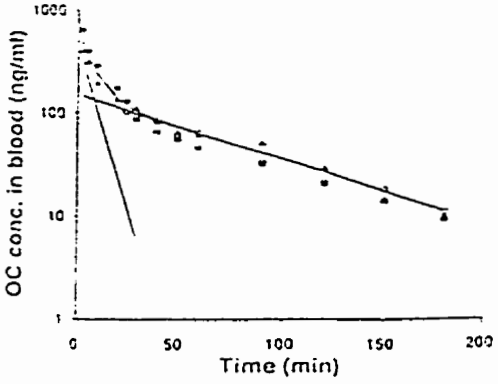

OC conc. in blood (ng/ml)
1000
100
10
1
0
50
100
150
200
Time (min)

Table 6. Pharmacokinetic parameters of OA and its analogs following intravenous administration of toxin (100 μg per rat weight 300 g)

Parameter[b]	Toxin[a]					
	OA	OP-OA	Oα	OA-OH	OB	OC
Blood						
No. of rats	6	6	3	3	3	3
t½α (min)	160±17	163±5	31±5	19±4.7	14±4	6±1.2
t½β (h)	103±16	50.5±2.8	9.6±2.3	6±0.9	4.2±1.2	0.6±0.2
k_{10} (1/min)	0.00032	0.00096	0.00462	0.00648	0.00608	0.04357
Vc (ml/kg)	162	62	144	167	118	604
Vp (ml/kg)	268	159	210	355	164	1885
$AUC_{0-\infty}$ (ng h/ml)	86386	98475	6680	4771	7625	233
Clearance						
No. of rats	9	9	6	6	6	
Cl_R (ml/h kg)	1.49±0.4	0.78±0.3	20.7±1.4	16.9±2.1	22.0±8.1	-
Cl_B (ml/h kg)	0.27±0.04	1.26±1.1	1.72±0.5	26.7±5.3	8.7±0.04	-
Cl_M (ml/h kg)	1.35	1.53	17.5	21.3	12.4	-
Cl_T (ml/h kg)	3.11	3.57	39.9	64.9	43.1	-

[a] Values are mean ± SD;

[b] t½α = distribution half-life; t½β = elimination half-life; k_{10} = the overall toxin elimination rate constant; Vc = volume of central compartment; Vp = volume of peripheral compartment; $AUC_{0-\infty}$ = the area under the curve of plasma concentration vs time from t = 0 to t = ∞ after toxin administration; Cl_T = total body clearance; CL_R = renal clearance; Cl_B = biliary clearance and Cl_M = metabolic clearance.

rats (2.1 h; Galtier et al., 1979), rabbits (1.9 h; Galtier et al., 1981) and cattle (1.8 h; Sreemannarayana et al., 1988), but not with chickens (0.5 h; Galtier et al., 1981). These data suggest that OA is relatively slowly distributed between the central and peripheral compartments of most animals. OP-OA, a newly discovered metabolite of OA (Xiao et al., 1996a), also had a distribution half-life similar to that of OA ($t_{1/2}\alpha$, OP-OA=163 min). The other metabolites of OA or OB had much shorter distribution half-lives with the $t_{1/2}\alpha$ values being 31, 19 and 14 min for Oα, OA-OH and OB, respectively. Comparative values for these other forms of OA are not available in the literature.

The elimination of OA and its metabolites from the blood is expressed as the elimination or a disappearance half-life ($t_{1/2}\beta$, Table 6). The elimination half-life of OA ($t_{1/2}\beta$ = 103 h) was much longer than that of its metabolites ($t_{1/2}\beta$ < 10 h) as the half-lives of Oα, OA-OH, and OC were 9.6, 6.0, and 0.6 h, respectively. Hagelberg et al. (1989) obtained $t_{1/2}\beta$ values for rats of 120 h for iv injected OA and 170 h for peripherally administered OA. Galtier et al. (1979) reported a value of 55 h for either oral or iv administration while Ballinger et al. (1986) reported a value of 56 h. Recently Breitholtz-Emanuelsson (1995) reported a value of 127 h for OA. All of these studies were with rats. Large differences in the blood half-life of OA in other species after oral or iv administration of OA have been reported with the values being 72-150 h for the pig (Galtier et al, 1981; Hagelberg et al., 1989), 77 h in the pre-ruminant calf, 24-48 h in the mouse (Fukui et al., 1987; Hagelberg et al., 1989), 8.2 h in rabbits (Galtier et al., 1981), 6.7-12 h in quail (Hagelberg et al., 1989), 4.1 h in chickens

(Galtier et al., 1981) and 0.7 to 8.3 h for fish (Hagelberg et al., 1989). The prolonged elimination half-life obtained for OA with the first few classes of animals including the rat suggests significant reabsorption and redistribution via enterohepatic circulation (Hagelberg et al., 1989; Fuchs and Hult, 1992; Sreemannarayana et al.,1988).

The elimination half-life of the toxic open form of OA was slightly less than half that of OA ($t_{1/2}\beta$=50.5 h for OP-OA), whereas the elimination half-lives of the nontoxic metabolites (Oα and OA-OH, Chu, 1974) and the much less toxic dechloro form of OA (OB, Chu, 1974) were more than 10 times lower (see Table 6) than that of OA. Hagelberg et al. (1989) reported $t_{1/2}\beta$ values for OB of 12 h for i.v. treated rats and 18 h for orally treated rats which compares with a $t_{1/2}\beta$ value of 14 h obtained in this study. Corresponding literature values for Oα and OA-OH, as far as the authors are aware of have not been reported. These results suggest that there is a positive relationship between the toxicity of the ochratoxins and their half-life values. The total clearances of OA, OP-OA, Oα, OA-OH and OB were 3.1, 3.6, 40, 65 and 43.1 ml/h kg, respectively. Therefore, processes that accelerate the conversion of OA to Oα such as an enhanced recycling in the bile which results in added exposure of OA to the hydrolytic action of intestinal microorganisms or enhanced conversion of OA to OA-OH in the P-450 system in the body (Marquardt and Frohlich, 1992) should result in an enhanced elimination of OA and therefore reduced toxicity.

Clearance Studies: The biliary (Cl_B), renal (Cl_R) and estimated metabolic (Cl_M) clearances of OA were much lower than those of the less toxic metabolites (Table 6). OA and

OP-OA, for example, had very low total clearance (Cl_T) with the rates being from 11 to 18 fold less than those of Oα, OA-OH and OB. A similar pattern was also observed with the toxic open form of OA (OP-OA). In general there was a close inverse relationship between the clearances of the different compounds and their corresponding half-life values as given in the previous section.

Clearance data suggest significant differences among the various forms of OA with regard to the preferred route(s) of elimination (renal, biliary and/or metabolism). OA, OB and Oα were mainly cleared via the urine (48% for OA, 51% for OB, and 52 % for Oα), whereas the hydroxylated form of OA (OA-OH) which was excreted mainly in the bile (41%). Nevertheless, a considerable amount of OA-OH appeared in the urine (26%) and as metabolites of OA-OH (33%). The open form of OA (OP-OA) was excreted mainly as metabolites (43%). Likewise OB, the dechloro form of OA, was also extensively metabolized (29%).

It is well known that OA is metabolized to its hydroxyl forms by the mixed function oxidase system located in the liver, kidney, other tissues and is hydrolyzed to its isocoumarin moiety by intestinal microorganism (see Marquardt and Frohlich, 1992 for a review). The nature of the excretory products, other than that of Oα, were not identified in our study. Storen et al. (1982) reported that 13% of the total OA plus OA-OH that was excreted in the urine was as OA-OH (4R). They also demonstrated that a considerable amount of OA was excreted as Oα but did not report on the excretion of other metabolites. Also they did not

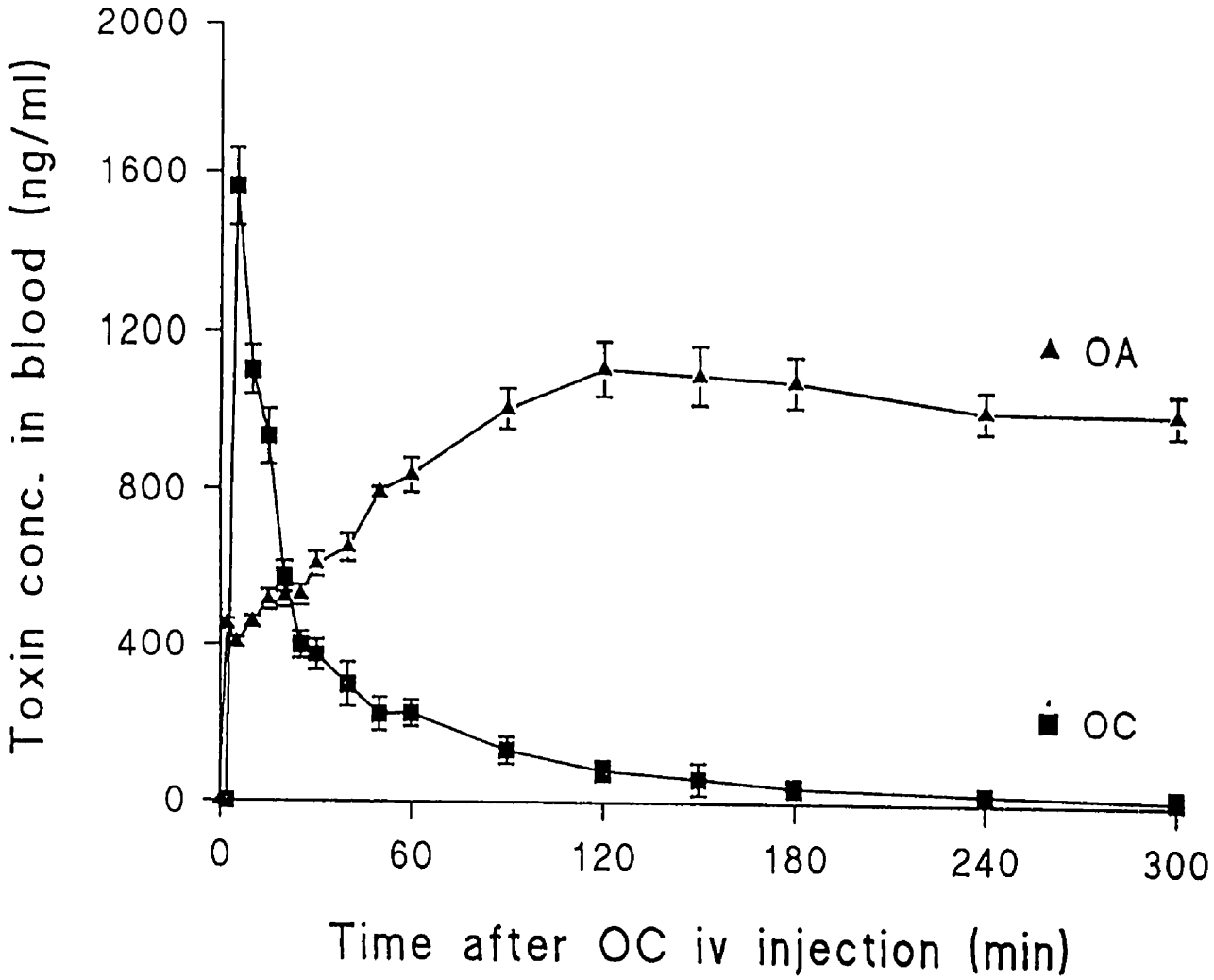
analyze fecal samples for any of the metabolites. Presumably a considerable amount of these compounds are also excreted in the feces. In the current study no Oα, as a metabolite, was detected in either the bile or urine (data not shown). These results would be expected as the intact ochratoxins would not have been subjected to hydrolysis in the gastrointestinal tract as bile was collected before it was secreted into the intestinal contents, the site where most of the OA is hydrolyzed to Oα (Madhyastha et al., 1992a). Madhyastha et al. (1992a) also demonstrated that up to 38% of the OA plus Oα that was excreted in the urine plus feces was excreted as Oα. They suggested that most of the Oα that was hydrolyzed by the microorganisms in the digesta was reabsorbed and then excreted in the urine. The collection of bile in this study, in addition to preventing the hydrolysis of OA, also eliminated the enterohepatic recycling of OA which, in turn, would have resulted in lower clearance values than would have been obtained under natural conditions.

Although the nature and amount of metabolites formed in the urine and especially the bile were not identified, HPLC analysis demonstrated the presence of 18 new peaks, some of which appeared to account for a significant portion of the total toxin that was administered (data not shown). It is conceivable that some of the metabolites in the bile were conjugates of OA or its metabolites (Roth et al., 1988) or further breakdown products of the parent compounds. These data, therefore, suggest that a considerable number of metabolites or conjugates are formed in the rat from OA and OB, or from metabolites of OA and that they represent alternate means for their disposition. Prior biotransformation of OA to OA-OH, Oα

and other compounds would greatly facilitate the excretion of OA since clearance of these compounds is much faster than that of OA. Overall, the results demonstrated that there are differential rates and sites of clearance of ochratoxin A and B and their metabolites, and that several metabolites of OA and OB are produced in the body. Similar clearance studies have not been reported in the literature.

Studies with OC: A typical blood concentration-time curve for OC (the ester form of OA), and for OA, after a bolus single iv injection of OC is shown in Fig 16. OC, which is a more hydrophobic form of the toxin, presumably facilitate delivery of the toxin to the cell but itself is rapidly converted to OA. Data in this figure indicated that approximately 90% of OC in the blood disappeared 1 h after injection and that there was a corresponding increase in the amount of OA in the blood. The calculated time for the toxin to decrease to 10% of its original value (i.e. 90% disappearance), as determined from its half-life value of 0.6 h (Table 6), was approximately 2 h which also demonstrates that OC is rapidly hydrolyzed to OA. Studies by Fuchs et al. (1984) have also indicated that OC was rapidly converted into OA in the body. They did not, however, report on the kinetics of this conversion. The results from the current study also suggests that the hydrolysis could have occurred in the peripheral tissues in addition to the blood since the distribution half-life of OC was much shorter than the elimination half-life, (6 min vs 0.6 h). The very low values for the distribution half-life of OC (6 min) compared to that of OA (160 min) also suggests that esterification of OA facilitated its delivery into the cell which in turn may affect maximal tissue concentrations and therefore

Fig 16. Typical blood concentration-time curve after a single iv injection of OC into three rats. Blood was collected after injection with OC at 2 and 5 min, at 5 min intervals up to 30 min, at 10 min intervals up to 60 min, at 30 min intervals up to 180 min and at 60 min up to 300 min as indicated in the figure. See Materials and Methods for further detail.


2000
1600
1200
800
400
0
Toxin conc. in blood (ng/ml)
▲ OA
■ OC
0
60
120
180
240
300
Time after OC iv injection (min)

its overall toxicity. This view is consistent with OC's extremely high value for Vp (1885 ml/kg), being three times its Vc (604 ml/kg). The magnitude of this effect, however, may not be large as the form of OA that was injected into rats (OA or OC) did not affect maximal concentrations of OA in the tissues (Table 6).

Tissue Distribution of OA: The results presented in Table 7 demonstrated the elimination half-life of OA in the different tissues was not influenced by the form of OA that was administered to rats; that is, similar results were obtained with both OC and OA. The half-life values for OA in liver, kidney and heart of OA-treated rats were similar (48 to 60h) and much lower than those of muscle (97 h) or blood (103 h, Table 6). Similar data were obtained for rats treated with OC. The reason for the more rapid rate of elimination of the toxin from the different tissues compared to blood was not established but it may be related, in part, to the binding affinity of the toxin to different proteins (Chang and Chu, 1977; Fuchs and Hult, 1992; Marquardt and Frohlich, 1992). The results of numerous studies suggest that the half-life of OA in the blood is directly related in part to the ability of plasma proteins to bind OA (Hagelberg et al., 1989). Presumably the binding affinity of plasma proteins is greater than that of tissue proteins and as a result the elimination half-life of OA is longer in blood than in tissues. These observations are consistent with the observation that the concentration of OA in blood is higher than that of the tissues (Mortensen, et al., 1983).

Conclusions: The half-life of OA in the blood was found to be 103 h. The half-lives of some of its metabolites were shorter being 50.5 h for OP-OA, 9.6 h for Oα, 6.0 h for OA-

Table 7. Half-life of OA in the tissue after a single intravenously administration of OA and OC in rats[a].

Tissue	OA treated rats		OC treated rats	
	C_{max}[b] of OA (ng/g)	Half-life of OA (h)	C_{max}[b] of OA (ng/g)	Half-life of OA (h)
Liver	280±10	60±10	355±63	59±13
Kidney	390±16	54±11	320±42	55±1.7
Muscle	190±10	97±8	125±7	91±3
Heart	290±16	48±12	275±35	57±6

[a] OA was administered to 16 rats OA (100 μg/rat) and 4 rats were killed at each of 4 time periods (2,24,48 and 96 h). OC (100 μg/rat) was administered to a similar number of rats for similar time periods.
[b] C_{max} = the maximum concentration of OA in the different tissue following OA or OC injection.

OH, 4.2 h for OB and 0.6 h for OC, respectively. The results suggest that biliary and renal excretion and metabolism were the major routes of elimination for OA. The renal (CL_R) and metabolic clearance (Cl_M) of OA were 1.49 and 1.35 ml/h kg, respectively, with the total clearance (CL_T) being 3.1 ml/h kg. Other clearance data are presented in the Table 1. No data was obtained for OC as it would have been difficult to obtain accurate clearance results in view its very short half-life (0.6 h). The results demonstrate that OA was very slowly cleared from the body and that its metabolites were cleared at a much faster rate. Its open form, OP-OA, was also cleared much more slowly than the metabolites. The different ochratoxins were also cleared to varying degrees via the bile and kidney and as metabolites. In general OA, Oα and OB were cleared mostly in the urine while OA-OH was cleared mostly in the bile. A specify amount of all of the ochratoxins were also cleared as metabolites. Treatments that enhance the conversion of OA to Oα or OA-OH in the animal would shorten its half-life and, therefore, its toxicity.

GENERAL DISCUSSION

Ochratoxin A is a nephrotoxic and carcinogenic mycotoxin that is widely distributed in the animal feedstuffs and human food in many countries including those in North America and Europe (Kuiper-Goodman and Scott, 1989; Marquardt, 1988; Ominski, 1996). Ochratoxin has been detected in food commodities in Manitoba and in human blood and mothers' milk (Frohlich et al., 1991; Ominski et al., 1996). The presence of OA in the food chain and its high toxicity can result in serious health effects and economic losses.

Several methods have been used for the detection of OA. HPLC is one of most widely used methods. Currently, HPLC methods are not suitable for the detection of metabolites of OA in tissues without a cleanup. The HPLC gradient elution used in this study could readily separate many of the different forms of ochratoxin in tissue samples without use of an elaborate sample cleanup procedure. This greatly facilitated the studies in this thesis and should be of benefit to researchers in the future. In addition there is a need for simple methods to confirm the presence of the ochratoxins in biological samples. Two simple procedures were developed in the current research to confirm the presence of ochratoxins and their metabolites in biological samples such as bile, urine, kidney, and liver. This involved their conversion into the methyl esters in methanol acidified with a strong acid or into the lactone opened forms in the presence of a strong base. The procedures developed in these studies can be used not only to identify OA and its metabolites but also can be employed to isolate and partially

characterize them. The esterification procedure is particularly useful and is much simpler than that developed by Nesheim et al. (1973). Also it does not result in production of other non-specific fluorescent peaks which occurs when the Nesheim et al. procedure is used. In addition the esterification method can be used for the synthesis of different esters of ochratoxins for use as reference standards.

The pharmacokinetic and clearance studies carried out in rats provided new and comprehensive information on the disposition of OA and its metabolites in animals. All of the toxins were distributed following a two compartment open model in a manner similar to that reported by Galiter et al. (1979) and Hagelberg et al. (1989) for OA. The results of this study are important as very little information is available in the literature on the half-life of OB and no data is reported for other forms of the ochratoxins including OA-OH, OC and Oα. Also there has been no reports on the clearance of any of the ochratoxins. A knowledge of the route of clearance of the ochratoxins will facilitate future studies on these compounds and will be useful in designing strategies to enhance their elimination from the body. The elimination half-lives of OA, OP-OA, Oα, OA-OH, OB and OC were 103 ± 16, 50.5 ± 2.8, 9.6 ± 2.3, 6 ± 0.9, 4.2 ± 1.2 and 0.6 ± 0.2 h, respectively. Total body clearance of OA, OP-OA, Oα, OA-OH and OB via the bile, urine and metabolic routes were 3.1, 3.6, 40, 65 and 43 ml/h kg, respectively. OA, OB and Oα were mainly cleared in the urine (≥48%), OA-OH in the bile (41%) and OP-OA mainly as metabolites (43%). Metabolism accounted for 43, 44, 33 and 29% of the total clearance of OA, Oα, OA-OH and OB, respectively. The results show that OA has a long half-

life and is very slowly cleared from the body and that of its metabolites are cleared at a much faster rate with much shorter half-lives. The newly discovered form of OA, OP-OA, also has a long half-life (50 h) and is highly toxic. The conversion of OA and OP-OA into their metabolites not only markedly enhances their rate of clearance from the body but also results in a greatly reduced level of toxicity. Therefore, procedures should be devised to enhance the conversion of OA and OP-OA in the body into Oα, OA-OH or other metabolites as this would shorten their half-lives and therefore their toxicity. The thesis discusses different strategies that can be used to do this. The research presented in this thesis resulted in the development of two procedures for the confirmation of different forms of OA, demonstrated that many metabolites of OA and its analogs are produced in the rat, established their half-lives and provided new information on the clearance of ochratoxins from the body.

CONCLUSIONS

Based on the research conducted, it can be concluded that:

1. OA, OB and OA-OH from tissue samples can be quantitatively converted (more than 90%) into their methyl esters in the presence of a high concentration of acid (6 or 12N HCl) and relatively large volumes of methanol (95%). The degree of conversion of Oα to its methyl ester, under optimal condition, was not quantitative but was as high as 72%. Also, under these conditions, there was a minimal degree of hydrolysis or decomposition of OA over time. Mass sepectrometry confirm that the new compounds were the methyl esters of the different ochratoxins. This procedure, therefore, provides a simple method for producing methyl esters of the ochratoxins.
2. All of the common ochratoxins can be converted into the lactone opened forms in the presence of a strong base and reformed at low pH values. This procedure provided a second method to confirm the presence of the different forms of the ochratoxins.
3. The ability to demonstrate that all of the different forms of the ochratoxins can be readily and reversibly interconverted into their methyl esters or lactone-opened forms provided a means of confirming the identity of the parent compounds as it is unlikely that coeluting contaminates would behave identically when treated in a similar manner. The present esterification method did not generate new fluorescent peaks in biological

sample and therefore avoids the problems associated with a previously used method. It was also shown that ethanol can replace methanol for esterification of the ochratoxins which in turn would result in the formation of ethyl esters of the different compounds. The esterification procedure can also be used to synthesize different esters of ochratoxin A for use as standards, etc.

4. There are many degradation products of OA and its metabolites in bile and urine of rats injected with OA and some of its metabolites. HPLC gradient analysis resulted in the separation more than 20 ochratoxins without a need for an elaborate sample cleanup procedure. Most of the metabolites were not identified.

5. The half-lives of OA and OP-OA in the blood of rats were found to be 103 and 50.5 h which was very slowly cleared from the body. The half-lives of their metabolites were shorter being 9.6 h for Oα, 6 h for OA-OH, 4.2 h for OB and 0.6 h for OC, respectively.

6. Biliary and renal excretion and metabolism were the major routes of elimination for ochratoxins. In general OA, Oα and OB were cleared mostly in the urine while OA-OH was cleared mostly in the bile. A considerable amount of all of the ochratoxins were also cleared as metabolites.

LITERATURE CITED

Abramson, D., R. N. Sinha, and J. T. Mills, 1980. Mycotoxin and odor formation in moist cereal grain during granary storage. Cereal Chem. 57:346-251.

Abramson, D., J. T., J. T. Mills, and B. R. Boycott. 1983, Mycotoxins and mycoflora in animal feedstuffs in western Canada. Can. J. Comp. Med. 47:23-26.

Abramson, D., 1991. Development of molds, mycotoxins and odors in moist creseals during storage. Pages 119-147, Elsevier Science Publishing Company INC. 655, Avenue of the Americas New York, NY 10010, U.S.A.

Abramson, D. J. T. Mills, R. R. Marquardt, and A. A. Frohlich, 1997. Myccotoxins in fungal contaminated samples of animal feed from western Canada, 1992-1994. Can. J. Vet. Res. 61:49-52.

Albassam, M. A., S. I. Yong, R. B. Bhatnagar, A. K. Sharma, and M. G. Prior, 1987. Histopathologic and electron microscopic studies on the acute toxicity of ochratoxin A in rats. Vet. Pathol. 24:427-435.

Appelate, K. L., J. R. chipley, 1973. Ochratoxins. Advances in appl. Microbiol. 16:97-109.

Appelgren, L.-E., and R. G. Arora, 1983. Distribution of ^{14}C-labelled ochratoxin A in pregnant mice. Food Chem. Toxicol. 21:563-568.

Arbid, M. S. S., M. S. Madhyastha, R.R. Marquardt, and A. A. Frohlich, 1993. Effect on neomycin on the hydrolysis and toxicity of vicine and convicine in rats. Food Chem. Toxicol. 11:835-840.

Bacon, C. W., J. G. Sweeney, J. D. Robins, and D. Burdick, 1973. Production of Penicillic acid and Ochratoxin A on poultry feed by *Aspergillus ochraceus*: Temperature and moisture requirements. App. Microbiol. 26:155-160.

Bailey, C. A., R. M. Gibson, L. F. Kubena, W. E. Huff, and R. B. Harvey, 1989. Ochratoxin A and dietary protein. 2. Effects on hematology and various clinical chemistry measurements. Poult. Sci. 68:1664-1671.

Bailey, C. A., R. M. Gibson, L. F. Kubena, W. E. Huff, and R. B. Harvey, 1990. Impact of

phenylalanine supplementation on the performance of three-week-old broilders fed diets containing ochratoxin A. 2. Effects on hematology and clinical chemistry. Poult. Sci. 69:420-425.

Baudrimont, I., R. Ahouandjivo, and E. E. Creppy, 1997a. Prevention of lipid peroxidation induced by ochratoxin A in vero cells in cuture by several agents. Chem. Biol. Interact. 104:29-40.

Baudrimont, I., A. M. Betbeder and E. E. Creppy, 1997b. Reduction of the ochratoxin A-induced toxicity in viro cells by aspartame. Arch. Toxicol. 71:290-298.

Ballinger, M. B., T. D. Phillips, and L. F. Kubena, 1986. Assessment of the distribution and elimination of ochratoxin A in the pregnant rat. J. Food Saf. 8:11-24.

Bendele, A., W. W. Carlton, P. Krogh, and E. B. Lillehoj, 1985. Ochratoxin A carcinogenesis in the (C57BL/6J x C3H)F1 mouse. J. Natl. Cancer Inst. 75:733-742.

Betina, V., 1984. Mycotoxins, production, isolation, separation, and purification. Pages 183-217, Elsevier Science Publishing Co., Inc., New York, NY.

Betina, V., 1989. Ochratoxins and related dihydroisocoumarins. Pages 151-173 in: Bioactive Molecules. Mycotoxins: Chemical, Biological and Environmental Aspects. Vol. No. 9 Elsevier, Amsterdam, The Netherlands.

Breitholtz-Emanuelsson, A., M. Olsen, A. Oskarsson, I. Palminger, K. Hult, 1993. Ochratoxin A in cow's milk and in human milk with corresponding human blood samples. J. AOAC Int. 76:842-846.

Breitholtz-Emanuelsson, A., F. Minervini, K. Hult, and A. Visconti, 1994. Ochratoxin A in human serum samples collected in southern Italy fron healthy individuals and individuals suffering from different kidney disorders. Nat. Toxins. 9:366-370.

Breitholtz-Emanuelsson, A., R. Fuchs, and K. Hult, 1995. Toxicokinetics of ochratoxin A in rat following intratracheal administration. Nat. Toxins. 3:101-103.

Bruinink, A., T. Rasonyi, and C. Sidler, 1997. Reduction of ochratoxin A toxicity by heat-induced epimerization. In vitro effects of ochratoxins on embryonic chick meningeal and other cultures. Toxicol. 118:205-210.

Burns, R. B., and P. Dwivedi, 1986. The natural occurrence of ochratoxin A and its effects in

poultry. A review. Part II. Pathology and immunology. World's Poult. Sci. J. 42:48-55.

CAST, 1989. Mycotoxins, Economic and Health Risks. Task Force Report No. 116, Council for Agricultural Science and Technology, Ames, IA.

Castegnaro, M, H. Bartsch, and I. Chernozemsky, 1987. Endemic nephropathy and urinary tract tumurs in the Balkans. Cancer Res. 47:3608-3609.

Castegnaro, M., H. Bartsch, J. C. Bereziat, P. Arvela, J. Michelon, and L. Broussolle, 1989. Polymorphic ochratoxin A hydroxylation in rat strains phenotyped as poor and extensive metabolizers of debrisoquine. Xenozebiotica. 19:225-230.

Chang, C. F., and F. S. Chu, 1977. The fate of ochratoxin A in rats. Food Cosmet. Toxicol. 15:199-204.

Chang, C. F., W. E. Huff, and P. B. Hamilton, 1979. A leucocytopenia induced in chickens by dietary ochratoxin A. Poult. Sci. 58:555-558.

Chang, C. F., J. A. Doerr, and P. B. Hamilton, 1981. Experimental ochratoxicosis in turkey poultry. Poult. Sci. 60:114-119.

Chelack, W. S., J. Borsa, R. R. Marquardt, A. A. Frohlich, 1991a. Role of competitive microbial flora in the radiation-induced enhancement of ochratoxin production by *Aspergillus alutaceus* var *aultaceus* NRRL-3147. Appl. Environ. Microbiol. 57:2492-2496.

Chelack, W. S., J. Borsa, G. Szekely, R. R. Marquardt, A. A. Frohlich, 1991b. Variants of *Aspergillus aultaceus* var *aultaceus* formerly *Aspergillus ochraceus*, with altered ochratoxin A production. Appl. Environ. Microbiol. 57:2487-2491.

Chelkowski, J., P. Golinski, B. Godlewska, W. Radomyska, K. Szebiotko, and M. Wiewiòrowska, 1981. Mycotoxins in cereal grains. Part IV. Inactivation of ochratoxin A and other mycotoxins during ammoniation. Nahrung. 25:631-637.

Christensen, C. M., F. A. Fanse, Nelson, G. H. Bates, and C. J. Mirocha, 1967. Microflora of black and red papers. Appl. Microbiol. 15:622.

Christensen, C. M., and H. H. Kaufman, 1969. Grain storage: The role of fungi in qulity loss. University of Minnesota Press, Minneapolis.

Chu, F. S., 1971. Interaction of ochratoxins with bovine serum albumin. Arch. Biochem. Biophys. 147:359-366.

Chu, F. S., I. Noh, and C. C. Chang, 1972. Structural requirements for ochratoxin intoxication. Life Sci. 11:503-508.

Chu, F. S., 1974. Studies on ochratoxins. CRC Crit. Rev. Toxicol. 2:499-524.

Chu, F. S., 1991. Mycotoxins: food contamination, mechanism, carcinogenic potencial and preventive measures. Mut. Res. 259:291-306.

Chu, F. S., 1992. Recent progress on analytical techniques for mycotoxins in feedstuffs. J. Anim. Sci. 70:3950-3963.

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

Cole, R. J., and R. H. Cox, 1981. Ochratoxins. Pages 128-151, in: Handbook of toxic fungi metabolites. Academic Press, New York.

Creppy, E. E., D. Kern, P. S. Steyn, R. Vleggaar, R. Röschenthaler, and G. Dirheimer, 1983a. Comparative study of the effect of ochratoxin A analogs on yeast aminoacyl-tRNA synthetases and on the growth and protein synthesis of hepatoma cells. Toxicol. Lett. 19:217-224.

Creppy, E. E., F. C. Størmer, D. kern, R. Röschenthaler, and G. Diheimer, 1983b. Effects of ochratoxin A metabolites on yeast phenylalaninyl-tRNA synthetase and on the growth and in vivo proteinsynthesis of hepatoma cells. Chem. Biol. Interact. 47:239-247.

Creppy, E. E., A. Kane, E. Giesen-Giouse, A. Roth, R. Röschenthaler, and G. Dirheimer, 1986. Effection of ochratoxin A on enzymes activities and micromolecules synthesis in MDCK cells. Toxicol. 9:310-314.

Creppy, E. E., K. Chakor, M. Jisher, and G. Dirheimer, 1990. The mycotoxin ochratoxin A is a substrate for phenylalanine hydroxylase in isolated rat hepatocytes and in vivo. Arch. Toxicol. 64:279-284.

Creppy, E.E., I. Baudrimont, and A. M. Betbeder, 1995. Prevention of nephrotoxicity of ochratoxin A, a food contaminat. Toxicol. Lett. 82/83:869-877.

de Scott, B., 1965. Toxigenic fungi isolated from cereal and legume products. Mycopath. Mycol. Appl. 25:213-222.

Dirheimer, G., and E. E. Creppy, 1991. Mechanism of action of ochratoxin A. Pages 171-186, in M. Castegnaro, R. Pleština, G. Dirheimer, I. N. Chernozemsky and H. Bartsch (Ed.) Mycotoxins, Endemic Nephrophay and Urinary Tract Tumours. Publ. Vol. No. 115 International Agency for Research on Cancer, Lyon, France.

Dirheimer, G., 1996. Mechanistic approaches to ochratoxin toxicity. Food Addit. Contam. 13:45-48.

Dörrenhaus, A., and W. Föllmann, 1997. Effects of ochratoxin A on DNA repair in cultures of rat hepatocytes and porcine urinary bladder epithelial cells. Arch. Toxicol. 71:709-713.

Doster, R. C., R. O. Sinnhuber, and J. H. Wales, 1972. Acute intraperitoneal toxicity of ochratoxin A and B in rainbow trout. Food Cosm. Toxicol. 10:85~92.

Doster, R. C., and R. O. Sinnhuber, 1972. Comparative rates of hydrolysis of ochratoxin A and B in vitro. Food Cosmet. Toxicol. 10:389-394.

Doupnik, B. JR., and J. C. Peckham, 1970. Mycotoxicity of *Aspergillus ochraceus* to chicks. Appl. Microbiol. 19:594-597.

Dwivedi, P., and R. B. Burns, 1984. Pathology of ochratoxicosis A in young broiler chickens. Res. Vet. Sci. 36:92-103.

Dwivedi, P., and R. B. Burns, 1986a. The natural occurrence of ochratoxin A and its effects in poultry: A review Part I. Epidemiology and toxicity. World's Poult. Sci. J. 42:32-47.

Dwivedi, P., and R. B. Burns, 1986b. The natural occurrence of ochratoxin A and its effects in poultry: A review. Part II. Pathology and immunology. World's Poult. Sci. J. 42:48-55.

Elling, F., B. Hald, C. Jacobsen, and P. Krogh, 1975. Spontaneous toxic nephropathy in poultry associated with ochratoxin A. Acta Pathol. Mycobiol. Scand. Sect. A, 83:739-741.

Elling, F. B., 1979. Ochratoxin A-induced mycotoxic porcine nephropathy: Alterations in

enzyme activity in tubular cells. Acta Pathol. Microbiol. Scand. Sect. A, 87:237-243.

Escher, F. E., P. E. Koehler, and J. C. Ayres, 1973. Production of ochratoxin A and B on country cured ham. Appl. Microbiol. 26:27-30.

Fink-Gremmels, j., M. J. Blom, N. FAM Woutersen-van, 1983. In vivo investigations on ochratoxin A metabolism. Pages 67-74, in E.E. Creppy, M. Castegnaro, G. Dirheimer (Ed.), Human ochratoxicosis and its Pathologies. Vol. No. 231, London: John Libbey Eurotext Ltd.

Fink-Gremmels, j., a. Jahn, and M. J. Blom, 1995. Toxicity and metabolism of ochratoxin A. Nat. Toxins. 3:214-220.

Frisvad, J. C., and R. A., Samson, 1991. Mycotoxins produced in species of *Penicillum* and *Aspergillus* occurring in cereals. Pages 441-476, in: J. Chelkowski (Ed.) Cereal Grain Mycotoxins, Fungi and Quality in Drying and Storage. Elsevier, Amsterdam, The Netherland.

Frohlich, A. A., Marquardt, R. R., and A. Bernatsky, 1988. Quantitation of ochratoxin A: Use of reverse phase thin-layer chromatography for sample cleanup followed by liquid chromatography or direct fluorescence measurements. J. Assoc. Off. Anal. Chem. 71: 949-953.

Frohlich, A. A., R. R. Marquardt, and K. H. Ominski, 1991. Ochratoxin A as a contaminant in the human food chain: A Canadian Perspective. Pages 139-143, in M. Castegnaro, R. Pleština, G. Dirheimer, I. N. Chernozemsky and H. Bartsch (Ed.) Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. International Agency for Research on Cancer, Lyon, France.

Frohlich A. A., R. R. Marquardt, and J. R. Clarke, 1997. Enzymatic and immunological approaches for the quantitation and conformation of ochratoxin A in swine kidneys. J. Food Prot. 60:172-176.

Fuchs, R., K. Hult, M. Peraica, B. Radic, and R. Plestina, 1984. Conversion of ochratoxin C into ochratoxin A in vivo. Appl. Environ. microbiol. 48:41-42.

Fuchs, R., L. E. Appelgren, and K. Hult, 1986. Distribution of ^{14}C-ochratoxin A in the rainbow trout (*salmo gairdneri*). Acta Pharmocol. Toxicol. 59:220-227.

Fuchs, R., L. E. Appelgren, and K. Hult, 1988a. Distribution of ^{14}C-ochratoxin A in the mouse

moniyored by whole body autoradiography. Pharmacol. Toxicol. 63:355-360.

Fuchs, R., L. E. Appelgren, S. Hagelberg, and K. Hult, 1988b. Carbon-14-ochratoxin A distribution in the Japanese Quail (*Coturnix coturnix japonica*) monitored by whole body autoradiography. Poult. Sci. 67:707-714.

Fuchs, R., B. Radić, M. Peraica, K. Hult, and Pleština, 1988c. Enterohepatic circulation of ochratoxin A in rats. Period. Biol. 90:39-42.

Fuchs, R., and K. hult, 1992. Ochratoxin A in blood and its pharmacokinetic preperties. Food chem. Toxicol. 30:201-204.

Fukui, Y., K. Hoshino, and Y. Kameyama, 1987. Placental transfer of ochratoxin A and its cytotoxic effect on the mouse embryonic brain. Food Cosmet. Toxicol. 25:17-24.

Frye, C. E., and F. S. Chu, 1977. Distribution of ochratoxin A in chicken tissues and eggs. J. Food Saf. 1:147-159.

Gibaldi, M., and D. Perrier, 1975. Pharmacokinetics. First Edition, Mercel Dekker, Inc., New York, 10016.

Galtier, P., and M. Alvinerie, 1976. In vitro transformation of ochratoxin A by animal microbial floras. Ann. Rech. Veter. 7:91-98.

Galtier, P., 1978. Contribution of pharmacokinetic studies to mycotoxicology-ochratoxin A. Vet. Sci. Commun. 1:349-358.

Galtier, P., J. -L. Charpenteau, M. Alvinerie, and C. Labouche, 1979. The pharmacokinetic profile of ochratoxin A in the rat after oral and intravenous administration. Drug Metab. Dispos. 7:429-434.

Galtier, P., M. Alvinerie, and J. L. Charpenteau, 1981. The pharmacokinetic profiles of ochratoxin A in pigs, rabbits and chickens. Food Cosmet. Toxicol. 19:735-738.

Galtier, P., 1991. Pharmacokinetics of ochratoxin A in animals. Pages 187-200, in M. Castegnaro, R. Plestina, G. Dirheimer, and I.N. Chemozemsky and H. Bartsch (Ed.), Mycotoxin Endemic Nephropathy and Urinary Tract Tumours, International Agency for Research on Cancer, Lyon, France.

Golinski, P., K. Hult, J. Grabarkiewicz-Szczesna, J. Chelkowski, and Piotr, 1984. Mycotoxin

ochratoxin A residues in kidney and blood of Polish swine. Appl. Environ. Microbiol. 47:1210-1212.

Golinski, P., K. Hult, J. Grabazkiewicz-Szczesna, J. Chelkowski, and K. Szebiotko, 1985. Spontaneous occurrence of ochratoxin A residues in porcine kidney and serum samples in Poland. Appl. Environ. Microbiol. 49:1014-1015.

Grosse, Y., I. Baudrimont, M. Castagnaro, A-M. Betbeder, E. E. Creppy and G. Dirheimer, 1995. Formation of ochratoxin A metabolites and DNA-adducts in monkey kidney cells. Chem-Biol. Interac. 95:175-187.

Hadidane, R., H. Bacha, E. E. Creppy, M. Hammami, F. Ellouze and G. Dirheimer, 1992. Isolation and structure determination of natural analogues of the mycotoxin ochratoxin A produced by *Aspergillus ochraceus*. Toxicol. 76:233-243.

Hagelberg, S., K. Hult, and R. Fuchs, 1989. Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. J. Appl. Toxicol. 9:91-96.

Hald, B., 1991. Porcine nephropathy in Europe. Pages 49-56 in: M. Castegnearo, R. Plestina, G. Dirheimer, I. N. Chernozemsky, and H. Bartzch (Eds.) Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. Publ. No. 115, International Agency for Research on Cancer, Lyon, France.

Hamilton, P. B., W. E. Huff, J. R. Harri, and R. D. Wyatt, 1982. Natural occurrences of ochratoxicosis in poultry. Poult. Sci. 61:1832-1841.

Hansen, C. E., S. Dueland, C. A. Drevon, and F. C. Stormer, 1982. Metabolism of ochratoxin A by primary cultures of rat hepatocytes. Appl. Environ. Microbiol. 43:1267-1271.

Hasinoff, B. B., A. D. Rahimtula, and R. F. Omer, 1990. NADPH-cytochrome-P-450 reductase promoted radical production by the iron (III)-ochratoxin A complex. Biochem. Biophys. Acta. 1036:78-81.

Hayes, M. A., R. D. Hood, and H. L. Lee, 1974. Teratogenic effects of ochratoxin A in mice. Teratol. 9:93-97.

Hayes, A., J. A. Cain, and G. Moore, 1977. Effect of aflatoxin Bl, ochratoxin A and Rubratoxin B on infant rats. Food Cosm. Toxicol. 15:23-27.

Hayes, A. W. (Ed.), 1981. Mycotoxin terogenecity and mutagenicity. CRC press, Boca Raton,

Florida.

Haazele, F. M., W. Guenter, R. R. Marquardt, and A. A. Frohlich, 1993. Beneficial effects of ditary ascorbic acid supplement on hens subjected to ochratoxin A toxicosis under normal and high ambientt temperatures. Can. J. Anim. Sci. 73:149-157.

Hoehler, D., R. R. Marquardt, A. R. McIntosh, and H. Xiao, 1996a. Free radical generation as induced by ochratoxin A and its analogs in bacteria (*Bacillus brevis*). J. Biol. Chem. 271:27388-27394.

Hoehler, D., R. R. Marquardt, 1996b. Influence of vitamins E and C on the toxin effects of ochratoxin A and T-2 toxin in chicks. Poult. Sci. 75:1508-1515.

Huff, W. E., J. A. Doerr, and P. B. Hamilton, 1979. Decreased glycogen mobilization during ochratoxicosis in broiler chickens. App. Environ. Microbiol. 37:122-126.

Huff, W. E., L. F. Kubena, and R. B. Harvey, and J. A. Doerr, 1988a. Mycotoxin interactions in poultry and swine. J. Anim. Sci. 66:2351-2355.

Huff, W. E., L. F. Kubena, and R. B. Harvey, 1988b. Progression of ochratoxicosis in broiler chickens. Poult. Sci. 67:1139-1146.

Hult, K., A. Teiling, and S. Gatenbeck, 1976. Degradation of ochratoxin A by a ruminant. Appl. Environ. Microbiol. 32:443-444.

Hult, K., E. Hökby, U. Hägglund, S. Gatenbeck, L. Rutqvist, and G. Sellyey, 1979. Ochratoxin A in swine blood: Method of analysis and use as a tool for feed studies. Appl. Environ. Microbiol. 38:772-776.

Hult, K., E. Hökby, S. Gatenbeck, and L. Rutqvist, 1980. Ochratoxin A in blood from slaughter pigs in sweden: Use in evaluation of toxin content of consumed feed. Appl. Environ. Microbiol. 39:828-830.

Hult, K., R. Pleština, V. Habazin-Novak, B. Radić, and S. Čevović, 1982. Ochratoxin A in human blood and Balkan endemic nephropathy. Arch. Toxicol. 51:313-321.

Hult, K., L. Rutqvis, T. holmberg, B. Thafvelin, and S. Gatenback, 1984. Ochratoxin A in blood of slaughter pigs. Nord. Vet. Med. 36:314-316.

Hutchinson, R. D., and P. S. Steyn, 1971. The isolation and structure of 4-hydroxyochratoxin

A and 7-carboxy-3, 4-dihydro-8-hydroxy-3-methyl-isocoumarin from *Penicillium viridicatum*. Tetrahedron Lett. 43:4033-4036.

IARC, 1993. Ochratoxin A. Monograph on the evaluation of carcinogenic Risks to Humans. Some naturally Occurring Food Items and Constituents, Heterocyclic Amines and Mycotoxins. 56:489-521.

Josefsson, E., 1979. Study of ochratoxin A in pig kidneys. Var. Foda. 31:415-420.

Josefsson, B. G. E., and T. E. Møller, 1980. Heat stability of ochratoxin A in pig products. J. Sci. Food Agric. 31:1313-1315.

Kane, A., E. E. Creppy, A. Roth, R. Röschenthaler, and G. Dirheimer, 1986. Distribution of the ^{3}H-label from low doses of radioactive ochratoxin A ingested by rats, and evidence for DNA single-strand breaks caused in liver and kidney. Arch. Toxicol. 58:219-224.

Kerkadi, A., C. Barriault, B. Tuchweber, 1998. Dietary cholestyramine reduces ochratoxin A-induced nephrotoxicity in the rat by decreasing plasma levels and enhancing fecal excretion of the toxin. J. Toxicol. Environ. Health. 53:231-250.

Khan, S., N. Matin, H. Bartsch, and D. Rahimtula, 1989. Perturbation of liver microsomal calcium homeostasis by ochratoxin A. Biochem. Pharmacol. 38:67-72.

Kiessling, K.-H., H. Pettersson, K. Sandholm, and M. Olsen, 1984. Metabolism of aflatoxin, ochratoxin, zearalenone, and trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. Appl. Environ. Microbiol. 47:1070-1073.

Kiessling, K. H., 1986. Biochemical mechanism of action of mycotoxins. Pure Appl. Chem. 58:327-338.

Kos, R., J. L. Stanley, M. T. Borin, 1991. Effect of competing anions on binding of bile salts by cholestyramine. Pharm. Res. 8:238-241.

Krogh, P., B. Hald, P. Gjertsen, F. Myken, 1974a. Fate of ochratoxin A and citrinin during melting and brewing experiments. Appl. Microbiol. 28:31-34.

Krogh, P., B. Hald, L. Englend, L. Rutqvist, O. Swahn, 1974b. Contamination of Swedish cereals with ochratoxin A. Acta Pathol. Microbiol. Scand. Sect, B. 82:301-302.

Krogh, p., 1976. Epidemiology of mycotoxic porcine nephropathy. Nord. Vet. Med. 28:452-

458.

Krogh, P., B. Hald, R. Pleština, and S. Ceović, 1977. Balkan (endemic) nephropathy and foodborn ochratoxin A: Preliminary results of a survey of foodstuffs. Acta Pathol. Mycobiol. Scand. Sect. B, 85:238-240.

Krogh, P., 1978. Casual associations of mycotoxic nephropathy. Acta Tathol. Microbiol. Acand. Sect. A. Suppl. 269:1-28.

Krogh, P., 1979. Environmental ochratoxin A and Balhan (endemic) nephropathy. Evidence for suport of a causal relationship. Pages 35-45, in S. Strahinijic and V. Stefanovic (Ed.), Endemic (Balkan) nephropathy, Nis. Yugoslavia.

Krogh, P., F. Elling, C.H.R. Friis, B. Hald, A. E. Larsen, E. B. Lillhoj, H. Madsen, P. Mortensen, F. Rasmussen, and U. Ravnskov, 1979. Porcine nepharopathy induced by long-term ingestin of ochratoxin A. Vet. Path. 16:456-475.

Krogh, P., 1983. Microbial nature and biological property of ochratoxins. Proceeding International Symposium, Mycotoxins. pp. 81-86.

krogh, P., 1987. Ochratoxins in Food. Pages 97-121, in P. Krogh (Ed.) Mycotcxins in Food, Academic Press, Harcourt Brace Jovanovich, London.

Krogh, P., N. Gyrd-Hansen, B. Hald, S. Larsen, J. P. Nielsen, M. Smith, C. Ivanoff, and H. Meisner, 1988. Renal enzyme activities in experimental ochratoxin A-induced porcine nephropathy: Diagnostic potential of phosphoenolpyruvaties carboxykinase and gamma-glutamyl transpetidase activity. J. Toxicol. Environ. Health. 23:1-14.

Krogh, P., 1992. Role of ochratoxin in disease caution. Food Chem. Toxicol. 30:213-224.

Kuiper-Goodman, T., and P. M. Scott, 1989. Risk assessment of mycotoxin ochratoxin A. Biomed. Environ. Sci. 2:179-248.

Kumagai, S., and K. Aibara, 1982. Intestinal absorption and excretion of ochratoxin A in rat. Toxicol. Appl. Pharmacol. 64:94-107.

Kumagai, S., 1985. Ochratoxin A: Plasma concentration and excretion onto bile and urine in albumin-deficient rats. Food Chemet. Toxicol. 23:941-943.

Kumagai, S., 1988. Effects of plasma ochratoxin A and luminal pH on the jejunal absorption

of ochratoxin A in rats. Food Chem. Toxicol. 26:753-758.

Lee, S. C., J. T. Beery, and F. S. Chu, 1984. Immunohistochemical fate of ochratoxin A in mice. Toxicol. Appl. Pharmacol. 72:218-227.

Levi, C. P., H. L. Trenk, and H. K. Mohr, 1974. Study of occurrence of ochratoxin A in green coffee beans. J. Asso. Off. Ana. Chem. 57:866-870.

Li, S., R. R. Marquardt, A. A. Frohlich, T. G. vitti, and G. Grow, 1997. Pharmacokinetics of ochratoxin A and its metabolites in rats. Toxixicol. Appl. Pharmacol. 145:82-90.

Madhyastha, M. S., R. R. Marquardt, and A. A. Frohlich, 1992a. Hydrolysis of ochratoxin A by the microbial activity of digesta in the gastrointestinal tract of rats. Arch. Environ. Contam. Toxicol. 23:468-472.

Madhyastha, M. S., R. R. Marquardt, and A. A. Frohlich, 1992b. Effect of dietary cholestyramine on the elimination pattern of ochratoxin A in rats. Food Chem. Toxicol. 30:709-714.

Madsen, A., H. P. Mortensen, and B. Hald, 1982. Feeding experiments with ochratoxin A contaminated barley for bacon pigs. 1. Influrence on pig performance and residues myrotoxin, residues in meat. Acta. Agric. Acand. 32:225-239.

Madsen, A. H. P. Hald, and H. P. Mortensen, 1983. Feeding experiments with ochratoxin A contaminated barley for bacon pigs. 1. Detoxification by ammonation heating + NaOH, or autoclaving. Scand. Assoc. Agric. Sci. 33:171-175.

Malaveille, C., G. Brun, and H. Bartsch, 1994. Structure-activity studies in E. coli strains on ochratoxin A (OTA) and it analogues implicate a genotoxic free radical and a cytotoxic thiol derivative as reactive metabolites. Mut. Res. 304:141-147.

Manning, R. O., and R. D. Wyatt, 1984. Toxicity of *Aspergillus ochraceus* contaminated wheat and different chemical forms of ochratoxin A in broiler chicks. Poul. Sci. 63:458-465.

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

Marquardt, R. R., and A. A. Frohlich, 1990. Ochratoxin A: an important western Canadian

storage mycotoxin. Can. J. Physiol. Pharmacol. 68:991-999.

Marquardt, R. R., and A. A. Frohlich, 1992. A reveiw of recent advances in understanding Ochratoxcosis. J. Anim. Sci. 70:3968-3988.

Meisner, H., and S. Chan, 1974. Ochratoxin A, an inhibitor of mitocondrial transport. Biochem. 13:2795-2800.

Meisner, H., and P. Krogh, 1986. Phosphoenolpryruvate carboxykinase as a selective indicator of ochratoxin A induced nephropathy. Dev. Toxicol. Environ. Sci. 14:199-291.

Meisner, H., and P. Meisner, 1981. Ochratoxin A, an in vivo inhibitor of renal phosphoenolpyruvate carboxykinase. Arch. Biochem. Biophys. 208:146-153.

Micco, C., M. Miraglia, R. Onor, A. Ioppolo, and A. Mantovani, 1987. Long term administration of low dose of mycotoxin in poultry. residues of ochratoxin A in broilers and laying hens. Poult. Sci. 66:47-50.

Munro, I. G., P. M. Scott, C. A. Moodie, and R. F. Willes, 1973. Ochratoxin A occurrence and toxicity. J. Amer. Vet. Med. Assoc. 163:1269-1273.

Moore, J. H., and B. Truelove, 1970. Ochratoxin A, inhibition of mitochondrial respiration. Science (Washington DC). 168:1102-1103.

Moroi, K., S. Suzuki, T. Kuga, M. Yamazaki, and M. Kanisawa, 1985. Reduction of ochratoxin A toxicity in mice treated with phenylalanine and phenobarbital. Toxicol. Lett. 25:1-5.

Mortensen, H. P., B. Hald, and A. Madsen, 1983. Feeding experiments with ochratoxin A contaminated barley for bacon pigs-5. Ochratoxin A in pig blood. Acta Agric. Scand. 33:235-239.

Mulder, G. J., E. Scholtens, and K. F. Meijer, 1981. Collection of metabolites in bile and urine from the rat, Detoxification and Drug Metabolism: Conjugation and Related Systems. Pages 21-30, in W. B. Jakoby, (Ed.), Method in Enzymology, Vol No, 77. Academic Press, NY.

Nel, W., and I. F. H. Purchase, 1968. The fate of ochratoxin A in rats. J. South Afri. Chem. Inst. 21:87-88.

Nesheim, S., 1969. Isolation and purification of ochratoxin A and B and preparation of their methyl and ethyl esters. J. Assoc. Offic. Anal. Chem. 52:975-979.

Nesheim, S., 1973. Analysis of ochratoxin A and B and their esters in Barley, using partition and thin Layer Chramatography. I. Development of the method. J. AOAC. 56:817-821.

Nesheim, S., 1976. The ochratoxins and other related compounds. Adv. Chem. Ser. 149:276-295.

Northolt, M. D., H. P. van Egmond, and W. E. Paulsch, 1979. Ochratoxin A production by some fungal species in relation to water activity and temperature. J. Food Prot. 42:476-484.

Omar, R. F., B. B. Mejilla, and A. Rahimtula, 1990. Mechanism of ochratoxin A stimulated lipid peroxidation. Biochem. Pharmacol. 40:1183-1191.

Omar, R. F., A. D. Rahimtula, and H. Bartsch, 1991. Role of cytochrome P-450 in ochratoxin A-stimulated lipid peroxidation. J. Biochem. Toxicol. 6:203-209.

Ominski, K. H., A. A. Frohlich, R. R. Marquardt, G. Crow, and D. Abramson, 1996. The incidence and distribution of ochratoxin A in Western Canadian swine. Food Addit. Contam. 13:185-198.

Osborne, B. G, 1980. The occurrence of ochratoxin A in mouldy bread and flour. Food Cosmet. Toxicol. 18:615-617.

Patterson, D. S. P., B. A. Roberts, and B. J. Small, 1976. Metabolism of ochratoxin A and B in the pig during early pregnancy and the accumulation in body tissues of ochratoxin A only. Food Cosmet. Toxicol. 14:439-442.

Pavlovic, M., R. Plestina, and P. Krogh, 1979. Ochratoxin A contamination of foodstuffs in an area with blakan (endemic) nephropathy. Acta. Path. Microbiol. Scand. Sect. B. 87:243-246.

Peckham, J. C., B. Doupnik and O. H. JR. Jones, 1971. Acute toxicity of ochratoxin A and B in chicks. Appl. Microbiol. 21:492-494.

Pepeljnjak, S., and Z. Cvetnic, 1985. The mycotoxicological chain and contamination of food by ochratoxin A in the nephropathic and non-nephropathic area in Yugoslavia. Mycpathol. 90:147-153.

Peterson R. E., and A. Ciegler, 1978. Ochratoxin A: isolation and subseqent purification by high-pressre liquid chromatography [*Aspergillus ochraceus*, Fungi]. Appl. Environ. Microbiol. 36:613-614.

Petkova-Bocharova, T., I. N. Chernozemsky, and M. Castegnaro, 1988. Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary system tumours in Bulgaria. Food Addit. Contam. 5:299-301.

Pfohl-Leszkowicz, A., Y. Grosse, A. kane, A. Gharbi, I. Beaudrimont, S. Obrecht, E. E. Creppy, and G. Dirheimer, 1993. Is the oxidative pathway implicated in the genotoxicity of ochratoxin A. In E. E. Creppy, M. Castegnaro, G. Dirheimer (Eds.), Human ochratoxicosis and its Pathologies. London, Jhon Libbey Eurotext Ltd, Vol. 23, pp 177-187.

Pfohl-Leszkowicz, A., 1994. Ochratoxin A, ubiquitous mycotoxin contaminating human food. CRS Soc. Biol. Fil. 188:335-353.

Pitout, M. J., 1968. The effect of ochratoxin A on glycogen storage in the liver. Toxicol. Appl. Pharmacol. 13:299-306.

Pitout, M. J., 1969. The hydrolysis of ochratoxin A by some proteolytic enzymes. Biochem. Pharmacol. 18:485-491.

Pleština, R., 1996. Nephrotoxicity of ochratoxin A. Food Addit. Contam. 13:49-50.

Prior, M. G., and C. S. Sisodia, 1982. The effects of ochratoxin A on the immune response of Swiss mice. Can. J. Comp. Med. 46:91-95.

Radić, B., R. Fuchs, M. Peraica, and A. Lucić, 1997. Ochratoxin A in human sera in the area with endemic nephropathy in Croatia. Toxicol. Lett. 91:105-109.

Rahimtula, A. D., J. C. Bereziat, V. Bussacchini-Griot, and H. Bartsch, 1988. Lipid peroxidation as a possible cause of ochratoxin A toxicity. Biochem. Pharmacol. 37:4469-4477.

Richard, J. L., L. H. Tiffany, and A. C. Pier, 1969. Toxigenic fungi associated with stored corn. Mycopathologia et Mycologia Applicata 38:313.

Röschenthaler, R., E. E. Creppy, and G. Dirheimer, 1984. Ochratoxin A: On the mode of action of a ubiquitous mycotoxin. J. Toxicol.-Toxin Rev. 3:53-86.

Roth, A., K. Chakor, E. E. Creppy, A. Kane, R. Röschenthaler, and G. Dirheimer, 1988. Evidence for an enterohepatic circulation of ochratoxin A in mice. Toxicol. 48:293-308.

Roth, A., E. E. Creppy, A. Kane, H. Bacha, P. S. Steyn, R. Röschenthaler, and G. Dirheimer, 1989. Influence of ochratoxin B on the ochratoxin A inhibition of phenylalanyl-tRNA formation in vitro and protein synthesis in hepatoma tissue culture cells. Toxicol. Lett. 45:307-314.

Rotter. R. G.. A. A. Frohlich, and R. R. Marquardt, 1989a. Influence of dietary charcoal on ochratoxin A toxicity in leghorn chicks. Can. J. Vet. Res. 53:449-453.

Rotter, R. G., R. R. Marquardt, and A. A. Frohlich, 1989b. Ochratoxin A toxicity in growing chicks: Effect of supplement dietary phenylalanine. Nutr. Rep. Int. 40:1091-1100.

Rotter, R. G., R. R. Marquardt, and A. A. Frohlich, 1990. Ensiling as a means of reducing ochratoxin A concentration in contaminated barley. J. Food Sci. Agri. 50:155-166.

Rutqvist, L., N. E. Bjorklund, K. Hult, and S. Gatensbeck, 1977. Spontaneous occurrence of ochratoxin A residues in kidney of fattening pigs. Zentralbl. Veterinarmend. 24:402-408.

Rutqvist, L., N. E. Bjorklund, K. Hult, E. Hokby, and B. Carlsson, 1978. Ochratoxin A as the cause of spontaneous nephropathy in fattening pigs. App. Environ. Microbiol. 36:920-925.

Scott, De B., 1965. Toxigenic fungi isolated from cereal and legume products. Mycopath. Mycol. Appl. 25:213-222.

Scott, P. M., 1977. Penicillium Mycotoxins. Pages 283-291. In: J. T. Wyllie, C.G. Morehouse. (Ed.), Marcel Dekker, Mycotoxic Fungi, Mycotoxins, Mycotoxicosis, Inc, Volume one.

Scott, P. M., S. R. Kanhere, and R. Canela, 1991. Determination of ochratoxin A in meat by liquid chromatography. Prehrambeno. Tehnol. Biotechnol. Rev. 29:61-64.

Scott, P. M., 1998. Survey of Canadian human blood plasma for ochratoxin A. Food Addit. Contam. 96:555-562.

Shargel, L., and A. B. C. Yu, 1985. In: Apllied Biopharmaceutics and Pharmacokinetics. Second edition. Edited by L. Shargel and A.B.C. Yu. Appleton-Century-Crofts, East

Norwalk, CT. 06855.

Shlosberg, A., N. Elkin, M. Malkinson, U. Orgad, V. Hanji, E. Bogin, Y. Weisman, M. Meroz, and R. Bock, 1997. Several hepatopathy in geese and broilers associated with ochratoxin in their feed. Mycopathol. 138:71-76.

Shotwell, O. L., C. W. Hesseltine, and M. L. Goulden, 1969. Ochratoxin A: Occurrence as a natural contaminant of corn sample. Appl. Microbiol. 17:765.

Sokol, P., G. Ripich, P. D. Holohan, and C. R. Ross, 1988. Mechanism of ochratoxin A transport in kidney. J. Pharmacol. Exper. Therepeut. 246:460-465.

Sreemannarayana, O., A. Frohlich, T. G. Vitti, R. R. Marquardt, and D. Abramson, 1988. Studies of the tolerance and distribution of ochratoxin A in young calves. J. Anim. Sci. 66:1703-1711.

Sreemannarayana, O., A. Frohlich, T. G. Vitti, R. R. Marquardt, and D. Abramson, G. D. Phillips, 1989. Organ weights, liver constituents, and serum components in growing chicks fed ochratoxin A. Arch. Environ. Contam. Toxicol. 18:404-410.

Stein, A. F., T. D. Philips, L. F. Kubena, and R. B. Harvey, 1985. Renal tubular secretion and secretion and reabsorption as factors in ochratoxicosis: Effects of probenecid on nephrotoxicity. J. Toxicol. Environ. Health. 16:593-605.

Steyn, P. S., and C. W. Holzapfel, 1967. The synthesis of ochratoxin A and B, metabolites of *Aspergillus ochraceus* Wilh. Tetrahedron. 23:4449-4461.

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

Steyn, P.S., R. Vleggaar, N.P. Dupreez, A.A. Blyth, J.C. Seegeras, the in vitro toxicity of analogs of ochratoxin A in monkey epithelial cells. Toxicol. Appl. Pharmacol. 32:198-203.

Steyn, P. S., 1971. Ochratoxin and other dihydroisocoumarins. Pages 179-205, In: A. Ciegler, S. Kadis and S. J. Ajl, (Ed.), Microbial Toxins: Vol. V. Fungal Toxins. Academic Press, New York.

Steyn P. S., 1984. Ochratoxins and related dihydroisocoumarins. Pages 183-216, In: V. Betina

(Ed.), Mycotoxins-Production, Isolation, Seperation and Purification, Elsevier Scientific Publishers, B. V. Amsterdam, The Netherlands.

Still, P. E., 1973. Mycotoxin as possible cause of abortion in dairy cattle. Ph.D. dissertation, The University of Wisconsin, Madison, Wisconsin.

Stojkvić, R., K. Hult, S. Gamulin, and K. Pleština, 1984. High affinity binding of ochratoxin A to plasma constituents. Biochem. Int. 9:33-38.

Størmer, F. C., and J. I. Pedersen, 1980. Formation of 4-hydroxyochratoxin A from ochratoxin A by rat liver microsomes. Appl. Environ. Microbiol. 39:971-975.

Størmer, F. C., C. E. Hansen, J. I. Pedersen, G. Hvistendall, and A. J. Aasen, 1981. Formation of (4R) and (4S)-4hydroxyochratoxin A from ochratoxin A by liver microsomes from various species. Appl. Environ. Microbiol. 42:1051-1056.

Størmer, F. C., O. Støren, C. E. Hansen, J. I. Pedersen, and A. J. Aasen, 1983. Formation of (4R)- and (4S)-4-hydroxyochratoxin A and 10-hydroxyochratoxin A from ochratoxin A by rabbit liver microsomes. Appl. Environ. Microbiol. 45:1183-1187.

Størmer, F. C., P. Kolsaker, H. Holm, S. Rogstad, and F. Elling, 1985. Metabolism of ochratoxin B and its possible effects upon the metabolism and toxicity of ochratoxin A in rats. Appl. Eviron. Microbiol. 49:1108-1112.

Størmer, F. C., 1992. Ochratoxin A - A mycotoxin of cancer. Pages 403-423, in D. Bhatnagar, E. B. Lillehoj, D. K. Arora (Ed.), Mycotoxins in Ecological Systems. Handbook of Applied mycology. Vol. V, New York, Marcel Dekker Inc.

Støren, O., H. Holm, and F. C. Størmer, 1982. Metabolism of ochratoxin A by rats. Appl. Environ. Microbiol. 44:785-789.

Subramanian, S., A. Kanthasamy, N. Balasubramanian, N. Sekar, and S. Govindasmy, 1989. Ochratoxin A toxicity on carbohydrate metabolism in rats. Bull Environ. Contam. Toxicol. 43:180-184.

Szczech, G. M., W. W. Carlton, J. Tuite, and R. Caldwell, 1973. Ochratoxin A toxicosis in swine. Vet. Pathol. 10:347-364.

Suzuki, S., T. S. Kozuka, and M. Yamazaki, 1975. Studies in the nephrotoxicity of ochratoxin A in rats. Toxicol. Appl. Pharmacol. 34:479-490.

Suzuki, S., T. Satoh, and M. Yamazaki, 1977. The pharmacokinetics of ochratoxin A in rats. Japan J. Pharmacol. 27:735-744.

Smyth D. D., and S. B. Penner, 1995. Renal I1-imidazoling receptor-selective compounds mediate natriuresis in the rat. J. Cardiovasc. Parmacol. 26:63-67.

Terao, K. and Y. Ueno, 1978. Morphological and function damage to cells and tissues. pp 189-238. In K. Uraguchi and M. Yamazaki (Eds.). Toxicology, biochemistry and pothlogy of mycotoxins. Halsted press, New York.

Uchiyama, S., and Y. Saito, 1987. Protein-binding potential of ochratoxin A in vitro and its fluorescence enhancement. J. Food Hyg. Soc. Japan. 28:453-460.

Udagawa, S., M. Ichinoe,and H. Kurata, 1970. Occurrence and distribution of mycotoxin producers in Japanese food, in Toxic Microorganism. Edited by M. Herzberg. UJNR-USDI. Washington, D.C. p 174.

Ueno, Y.. 1984. The toxicology of mycotoxins. CRC Crit. Rev. Toxicol. 14:99-132.

Ueno, Y., O. Sugiura, K. Horiguchi, M. Najima, K. Yamamoto, and S. Sato, 1991. Use of monoclonal antibodies, enzyme-linked immunosorbent assay and immunoaffinity column to determin ochratoxin A in porcine sera, coffee products and toxin-producing fungi. Pages 71-75 in: Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. M. Castegnaro, R. Plestina, G. Dirheimer, I. N. Chernozemsky, and H. Bartsch (Eds.). Publ. No. 115, International Agency for Research on Cancer, Lyon, France.

Unglaub, W. and F. Holl, 1990. Bestimmung und Gehalte von ochratoxin A in Schweineblut und - seren. Fleischwirtschaft. 70:406-

Van Der Merwe, K. J., P. S. Steyn, L. Fourie, D. B. Scoot, and I. J. Theron, 1965a. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh,. Nature 205:1112-1113.

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

Viscontin, A., and P. B. Bottalico, 1983. High levels of ochratoxin A and B in mouldy bread responsible for mycotoxicosis in farm animals. J. Agri. Food Chem. 31:1122-1123.

Wallace, H. A. H., and R. N. Sinha, 1962. Fungi associated with hot spots in farm stored grain. Can. J. PLant Sci. 42:130.

Warren, M. F., and P. B. Hamilton, 1980. Inhibition of the glycogen phosphorylase system during ochratoxicosis in chickens. Appl. Environ. Microbiol. 40:522-525.

Wei, R. D., P. E. Still, E. B. Smalley, H. K. Schnoes, and F. M. Strone, 1973. Isolation and partial characterization of a mycotoxin from Penicillum roquefori. Appl. Microciol. 25.:111-114.

Wei, Y. H., C. Y. Lu, T. N. Lin, and R. D. Wei, 1985. Effect of ochratoxin A on rat liver mitochondrial respiration and oxidative phosphorylation. Toxicol. 36:119-130.

Williams, B. C., 1985. Mycotoxins in foods and foodstuffs. Pages 49-52, in: Mycotoxins: A Canadian Perspective. P. M. Scott, H. L. Trenholm, and M. D. Sutton (Eds.) National Research Council of Canada, Ottawa.

Xiao, H., R. R. Marquardt, A. A. Frohlich, G. D. Phillips, and T. G. Vitti, 1991a. Effect of a hay on and a grain diet on the hydrolysis of ochratoxin A in the rumen of sheep. J. Anim. Sci. 69:3706-3714

Xiao, H., R. R. Marquardt, A. A. Frohlich, G. D. Phillips, and T. G. Vitti, 1991b. Effect of a hay and a grain diet on the bioavailability of ochratoxin A in the rumen of sheep. J. Anim. Sci. 69:3715-3723..

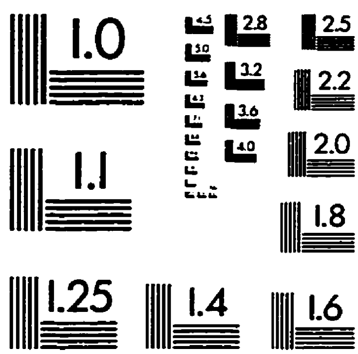
Xiao, H., R. R. Marquardt, A. A. Frohlich, and Z. Y. Ling, 1995. Synthesis and structural elucidation of analogs of ochratoxin A. J. Agric. Food Chem. 43:524-530.

Xiao, H., S. Madhyastha, R. R. Marquardt, S. Li, J. K. Vodela, A. A. Frohlich, and B. W. Kemppainen, 1996a. Toxicity of ochratoxin A, its opened lactone form and several of its analogs: structure-activity relationships. Toxicol. Appl. Pharmacol. 137:182-192.

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

Yamazaki, M., Y. Maebayashi, and K. Miyaki, 1970. Production of ochratoxin A by *Aspergillus ochraceus* isolated in Japen from moldy rice. Appl. Microbiol. 21:452-454.

IMAGE EVALUATION TEST TARGET (QA-3)



150mm

6"

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989