THE PHARMACOKINETICS OF OCHRATOXIN A IN RATS

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Abstract—The absorption and tissue distribution of ochratoxin A (OCT A) following a single oral dose of OCT A were investigated in adult, male Wistar rats. In experiments concerning excretory patterns of OCT A, 14C-OCT A was used. A relatively large amount of OCT A was found in the circulating blood 48 hours after dosing. The patterns of absorption, tissue distribution and excretion of OCT A were affected by acute catarrhal enteritis produced by OCT A and/or ochratoxin α (OCT α). Quantitative data show that OCT A is distributed mostly in the kidney and this finding is closely associated with the tissue specificity of OCT A-induced nephrotoxicity. OCT A was found to be hydrolyzed to its major metabolite, OCT α by addition of the homogenate of pancreas, duodenum and ileum. Approximately 56% of OCT A administered was excreted in both urine and feces as the unchanged toxin and OCT α during 120 hours following dosing. A relatively larger amount of OCT α was detected as compared with that of OCT A.

Ochratoxin A (OCT A) is a fungal metabolite first isolated from Aspergillus ochraceus Wilhelm (1) and later, identified as a metabolite of Penicillium viridicatum Westling (2) and other members of A. ochraceus group (3, 4).

Acute toxicity studies (5, 6) indicated that OCT A produced renal and hepatic changes in weanling rats when given in doses approximating the LD50. Short-term and subacute studies (7, 8) indicated that the hepatic changes in the treated rats were not marked and suggested that in rats, OCT A is primarily nephrotoxic. Suzuki and Satoh (9) demonstrated that the remarkable depletion of hepatic glycogen became evident only 4 hr after oral dosing of OCT A (15 mg/kg). In a previous study, Suzuki et al. (10) reported that three consecutive doses of OCT A (5 or 15 mg/kg) induced significant thickening of the basement membrane in the proximal tubules, and such was similar to findings in chronic studies reported by Munro et al. (7) and Kanisawa et al. (8). Kanisawa et al. (11) also reported that severe catarrhal enteritis in the small intestine was observed within 2 to 4 hr after a single dosing of OCT A at doses over 3 mg/kg. With a single dose, toxicological manifestations appeared mainly in the small intestine and with a single large or repeated small dose of OCT A, manifestation were noted in the kidney.

To our knowledge, an explanation of the mechanism of toxic action has not been documented.

This report deals with the distribution of OCT A in liver and other tissues of rats during the 24 or 48 hr period following oral dosing of unlabelled OCT A and the excretion patterns

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of radioactivity of labelled OCT A and OCT α over 120 hr after a single dosing of labelled OCT A in rats.

MATERIALS AND METHODS

Absorption and distribution of OCT A

OCT A was produced by A. ochraceus Wilh. (IFM 4443) by the method of Yamazaki et al. (12) and OCT α was obtained by hydrolysis of OCT A in a concentrated hydrochloric acid according to the method of Van der Merwe et al. (13). OCT A and OCT α were purified and quantified as described in a previous paper (10).

Three or more adult, male Wistar rats weighing 200–250 g were given OCT A p.o. at a dose of 15 mg/kg and the compound was dissolved in 0.1 M sodium bicarbonate (NaHCO₃) solution. Control rats were given 0.1 M NaHCO₃ solution. The volume was adjusted to 0.4 ml per 100 g body weight and animals were maintained on a standard commercial diet and provided water ad libitum. The time of administration of OCT A was fixed at 10:00 a.m., as absorption of OCT A depends to some extent on contents remaining in the stomach. The animals were anaesthetized with ethyl ether and sacrificed at time intervals of 1 to 48 hr after dosing. Liver, kidneys and heart were excised and weighed. The gastro-intestinal tract was also excised and separated into four, i.e., stomach, duodenum, ileum and colon. The content of each segment was removed by washing in ice-cold 1.15% potassium chloride solution. Two ml of blood withdrawn from the abdominal aorta, 2 g of liver and whole amounts of other tissues were used for analysis of OCT A content. These materials were homogenized in 2 volumes of 10% trichloroacetic acid (TCA) using a glass homogenizer. The homogenate thus obtained was diluted with 4-fold of water. OCT A and OCT α in these homogenates was extracted with a hundred-fold of chloroform (CHCl₃). These preparations were shaken for 30 min using a mechanical shaker. The CHCl₃ layer was pooled and evaporated to dryness in vacuo. The residue was dissolved again in an appropriate volume of CHCl₃, then 5 or 10 μl aliquots of the resultant CHCl₃ solution, OCT A standard solution (25 and 150 ng/spot) and OCT α standard solution (150 ng/spot) were chromatographed on thin-layer chromatoplate of silica gel G (Merck) with benzene-acetic acid (9:1) as the mobile phase. The spots of OCT A and OCT α were detected under UV light. In addition, positive findings of residues were confirmed by the methods of Nel and Purchase (14), and Nesheim et al. (15). The concentration of OCT A on the plate was measured by spectrophotofluorodensitometry (16). The relationship between the concentration and fluorescence intensity of OCT A in this procedure is linear between 12.5 to 150 ng/spot for the standard. Throughout the experiments, including the extraction procedure, the recovery of the added OCT A standard in the blood or tissues was 64% for blood, 75% for liver, 69% for kidney, 74% for heart, 84% for stomach, 91% for duodenum, 83% for ileum and 74% for colon, respectively with a lower detection limit of 1 μg/g. The data have been corrected to 100% recovery.

Excretion of ¹⁴C-OCT A

Radioactive OCT A, labelled in the isocoumarin moiety (Fig. 1) was produced in this
institute by isolation from *A. ochraceus* (IFM 4443) cultures supplemented with sodium malonate-2-14C according to the method of Yamazaki *et al.* (17). The crystalline material moved as a single substance on silica gel G plates (Merck) and contained all radioactivity detectable by autoradiography of the chromatograms on X-ray film (Fuji Film Co.). The specific activity of 14C-OCT A was 3.3 nCi/μmole.

**Determination of radioactivity in the feces and urine**

In the experiments with radioactive OCT A, male Wistar rats weighing about 240 g were given orally at 10:00 a.m. 3.6 mg (29.5 nCi) of 14C-OCT A dissolved in 0.96 ml of 0.1 M NaHCO₃ solution per animal. Immediately after dosing, each animal was placed back into a cage that permitted quantitative, separate collection of feces and urine. Animals were also maintained as those used in the study of distribution. The feces were collected daily for a total of 120 hr. The urine was also collected in each period of 0–12, 12–24, 24–48, 48–72, 72–96 and 96–120 hr after dosing. Feces were homogenized with 10-fold of methanol in a VirTis homogenizer at 25,000 rpm for 5 min. The residue was filtered and rehomogenized, then filtered. The extract was pooled and evaporated to dryness in vacuo. The recovery of 14C-OCT A added to the feces was 50% in this extraction procedure. The dry residue was dissolved in 1 ml of methanol. An aliquot of the sample prepared above or urine (100–200 μl) was decolorized with hydrogen peroxide by the method of Bruno and Christian (18) and used for determination of the radioactivity after mixing with 15 ml of scintillator consisting of a mixture of 0.6% PPO (2,5-diphenyl-oxazole) in toluene and 2-methoxyethanol (1:1, V/V).

**Determination of radioactivity derived from 14C-labelled OCT A and OCT α**

Extraction was carried out twice with a sample of feces or urine, 50 ml of 0.1 M NaHCO₃ solution and 100 ml of CHCl₃ in a mechanical shaker for 30 min to extract both OCT A and OCT α. The NaHCO₃ layer of the extract was separated and adjusted to pH 3 with concentrated hydrochloric acid, after which the mixture was extracted with 100 ml of CHCl₃ twice. The solvent was evaporated to dryness in vacuo. The residue was dissolved in 1.0 ml of CHCl₃ and subjected to thin-layer chromatography (TLC) analysis. OCT A and OCT α in the resultant CHCl₃ solution were checked by the same method used in the study of distribution. The radioactivity in each spot was measured using liquid scintillation technique by scraping the spot from the plate and allowing it to dissolve in the toluene scintillator containing 0.6% PPO and 0.01% POPOP [1,4-bis 2-(5-phenyl-oxazole)benzene]. The radioactivity in the spot was almost completely recovered in this procedure. All radioactivity recovered from the feces and the urine was that of labelled OCT A and OCT α.

**Determination of radioactivity in the bile**

Male Wistar rats weighing about 250 g were given 4 mg (32 nCi) of 14C-OCT A dissolved
in 1.0 ml of 0.1 M NaHCO₃ solution per animal. In each animal, a cannula was inserted into the bile duct and the animal was fixed in a restraint cage to collect the bile in each period of 0-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-5 and 5-6 hr after dosing. Fifty μl of the bile collected was used for determination of the radioactivity using the same method as that applied in the case of feces and urine.

Hydrolysis studies of OCT A

The hydrolysis of OCT A was determined by homogenates of the liver, kidney, duodenum, ileum and pancreas from rats according to the method of Folk and Schirmer (19) but with minor modification. Four male Wistar rats (average body weight 250 g) were decapitated, the tissues were removed, washed with ice-cold 0.9% NaCl solution and weighed. These tissues were homogenized in 2 volumes of 10% lithium chloride solution in a glass homogenizer. The incubation mixture contained 3.75 ml of 0.05 M Tris-HCl buffer (pH 7.5), 0.25 ml of NaHCO₃ solution containing ¹⁴C-OCT A (744 nmoles, 2.46 nCi), 2.75 ml of redistilled water and 0.25 ml of homogenate from rat tissues. Control mixtures were similarly prepared except that they contained 0.25 ml of heat-denatured homogenate. All incubations were carried out in a metabolic shaker at 37°C for 6 hr. The reaction was stopped by the addition of 1.0 ml of 10% TCA. Extraction of OCT A and OCT α from the incubation mixtures and verification of identity of OCT A and OCT α were made by a procedure similar to that used for determination of radioactivity derived from ¹⁴C-OCT A and ¹⁴C-OCT α. Protein concentration was determined by the biuret method (20).

A Beckman LS-150 liquid scintillation spectrometer was used for measurement of radioactivity. The average counting efficiencies determined using an external standard were about 78%. The data have been corrected to 100% recovery.

RESULTS

Absorption and distribution of OCT A

Concentration of OCT A in the blood:

Fig. 2 shows the time course of the concentration of OCT A in μg per ml of the blood from the OCT A-treated rats. The level of OCT A in blood was initially high during the first 4 hr, and then decreased markedly at 6 hr followed by a gradual increase in 24 hr. The OCT A content lowered to 35% of the maximum level in blood in 48 hr after dosing.

Distribution of OCT A in the tissues:

The tissue distribution of OCT A in rats sacrificed at time intervals during the 48 hr period following oral dosing is shown in Fig. 3. The content of OCT A
FIG. 3. Tissue distribution of ochratoxin A following a single oral dose of ochratoxin A (15 mg/kg). Data from same animals as in Fig. 2. and explanation as in the same Fig.

Fig. 4. Distribution of ochratoxin A in the gastrointestinal tract following a single oral dose of ochratoxin A (15 mg/kg). Data from same animals as in Fig. 2. and explanation as in the same Fig.

A in μg per g wet weight of the tissues was plotted against time. The highest level of OCT A was found in the kidney in 4 hr, then decreased in 6 to 8 hr followed by an increase and then remained at a fairly constant level during 16 to 48 hr. Higher concentrations of OCT A were also noted in liver and heart at 4 hr. In these tissues, the distribution of OCT A varied in good parallel with values noted in the blood, from 8 to 48 hr after dosing of OCT A. The kidney contained greater amounts of OCT A than did the liver and heart at 4 or 48 hr. OCT A was also detected in the brain, lung, testis and adipose tissue, but the amount was insufficient for quantitative analysis. Little or no OCT α was detected qualitatively in the liver and kidney 48 hr after dosing. Apparently there is no accurate procedure for OCT α.

Distribution of OCT A in the gastrointestinal tract and acute enteritis in the small intestine:

The distribution of OCT A in various segments of the gastrointestinal tract of rats is illustrated in Fig. 4. The OCT A content in the stomach was high at 1 hr, then decreased rapidly at 2 hr and again increased to 65% of the maximum level at 4 hr followed by a gradual decrease in 24 hr. Higher concentrations of OCT A were quite evident in the duodenum as compared with the ileum and colon as early as 1 hr after dosing of OCT A, while, lower concentrations of OCT A were found in the ileum and colon, throughout the entire experimental period. OCT A was still detectable in the gastro-intestinal tract at 48 hr, but the amount was insufficient for a quantitative measurement.

OCT A produced marked catarrhal enteritis through the small intestine. Macroscopic signs of enteritis were observed during 1.5 to 8 hr. The dilated intestinal lumen was filled with excreted serous fluid and mucus during 4 to 8 hr, the amount measuring about 5 to 10 ml. Large amounts of OCT A in the excreted serous fluid and mucus were detected during 4 to 8 hr. The concentration of OCT A in the excreted serous fluid and mucus was 4.7 ± 0.7 μg/ml at 4 hr; 3.9 ± 1.2 μg/ml at 6 hr; 6.1 ± 2.2 μg/ml at 8 hr (expressed as means ± standard error). The OCT α was also detected in the excreted serous fluid and mucus,
although such was not analyzed quantitatively. The excreted serous fluid and mucus was absorbed completely at 16 hr after dosing of OCT A.

**Excretion of \(^{14}\text{C}-\text{OCT A}$$**

**Radioactivity excreted in the feces:** Table 1 summarizes the volume of feces obtained at each time interval and the excretion of radioactivity in the feces up to 120 hr after dosing of \(^{14}\text{C}-\text{OCT A}$$

The volume was initially small at the period between 0-24 hr, and then gradually recovered to a normal level (about 10 g/rat). Within 24 hr after dosing, 3.1% of administered radioactivity of \(^{14}\text{C}-\text{OCT A}$$ was excreted, and the excretion rate in the feces reached a maximum within 24-48 hr. About one-third of the radioactivity administered was excreted over a 120 hr period. Excretion of the radioactivity derived from \(^{14}\text{C}-\text{OCT a}$$, a major metabolite of \(^{14}\text{C}-\text{OCT A}$$, was twice as high as that derived from \(^{14}\text{C}-\text{OCT A}$$ in the feces during 24-120 hr.

**Radioactivity excreted in the urine:** Table 2 shows the urine volume and the excretion of radioactivity in the urine up to 120 hr after dosing of \(^{14}\text{C}-\text{OCT A}$$

Urine volume was relatively little up to 48 hr and gradually returned to the normal level (about 11 ml/rat). The excretion of radioactivity in the urine up to 24 and 120 hr after dosing was 6.1% and 22.4% of \(^{14}\text{C}-\text{OCT A}$$, respectively. The excretion of \(^{14}\text{C}-\text{OCT A}$$ radioactivity in the urine

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**Table 1.** Feces volume or fecal excretion of radioactivity in rats after administration of labelled Ochratoxin A*

| Period (hr) | Feces volume (g) | OCT A | OCT a | Total  |
|-------------|------------------|-------|-------|--------|
| 0-24        | 1.7±0.0          | 2.0±0.1| 1.1±0.0| 3.1±0.1|
| 24-48       | 6.8±1.9          | 5.5±0.0| 12.7±0.6| 18.2±0.7|
| 48-72       | 5.1±0.9          | 8.5±0.3| 17.8±1.0| 26.3±1.3|
| 72-96       | 7.5±0.3          | 9.6±0.4| 22.2±1.8| 31.8±1.4|
| 96-120      | 10.7±0.5         | 11.4±0.2| 22.9±1.8| 34.3±1.5|

* Values represent the means ± standard error of 3 animals.

**Table 2.** Urine volume or urinary excretion of radioactivity in rats after administration of labelled Ochratoxin A*

| Period (hr) | Urine volume (ml) | OCT A | OCT a | Total  |
|-------------|-------------------|-------|-------|--------|
| 0-12        | 1.5±0.5           | 1.0±0.4| 0.5±0.1| 1.5±0.5|
| 12-24       | 1.4±0.1           | 1.6±0.5| 4.5±0.9| 6.1±1.5|
| 24-48       | 1.8±0.2           | 4.6±0.6| 7.7±0.8| 12.4±1.3|
| 48-72       | 3.5±1.9           | 5.8±0.8| 10.9±1.4| 16.6±2.2|
| 72-96       | 7.4±1.3           | 8.3±0.9| 11.1±1.5| 19.4±2.3|
| 96-120      | 11.4±0.3          | 10.8±1.2| 11.6±1.6| 22.4±2.7|

* Explanation as in Table 1. Data from same animals as in Table 1.
TABLE 3. Bile volume or biliary excretion of radioactivity in rats after administration of labelled Ochratoxin A*

| Period (hr) | Bile volume (ml) | Cumulative bile excretion (Percent of dose administration) |
|------------|------------------|----------------------------------------------------------|
| 0-0.5      | 0.43±0.10        | 0.9±0.1                                                  |
| 0.5-1      | 0.47±0.00        | 5.0±0.3                                                  |
| 1-2        | 1.10±0.14        | 14.4±0.8                                                 |
| 2-3        | 1.20±0.17        | 21.2±2.0                                                 |
| 3-4        | 0.87±0.10        | 25.6±2.7                                                 |
| 4-5        | 0.90±0.10        | 29.3±3.1                                                 |
| 5-6        | 0.90±0.00        | 33.0±3.2                                                 |

* Explanation as in Table 1.

TABLE 4. Amount of Ochratoxin α formed from Ochratoxin A by homogenate of various rat tissues*

| Ochratoxin A in reaction mixture | Tissue     | Total ochratoxin α formed*** |
|----------------------------------|------------|-----------------------------|
| 744 nmoles                       | Liver      | Trace***                    |
|                                  | Kidney     | Trace***                    |
|                                  | Pancreas   | 1.7±0.4                    |
|                                  | Duodenum   | 1.4±0.1                    |
|                                  | Ileum      | 1.4±0.2                    |

* Values represent the means±standard error of 4 experiments.

** Expressed as nmoles ochratoxin α/mg protein of tissue homogenates/6 hr.

*** Less than 0.3 nmoles ochratoxin α.

reached a maximum within 24-48 hr, while the maximum excretion rate derived from 14C-OCT α radioactivity was seen within 12-24 hr. Excretion of the radioactivity derived from 14C-OCT α in the urine and feces was higher than that derived from 14C-OCT A during 12-72 hr, but increase of the radioactivity derived from 14C-OCT α in the urine was not observed during 72-120 hr. Approximately one-fifth of the administered dose was excreted over a 120 hr period. Total radioactivity in the fecal and urinary excretion was 56% of 14C-OCT A administered.

Radioactivity excreted in the bile: Table 3 demonstrates the time course of biliary excretion of the radioactivity up to 6 hr after dosing of 14C-OCT A. The radioactivity in the bile was high from 0.5 to 3 hr, and 33.0% of radioactivity of the administered dose was excreted into the bile up to 6 hr after dosing. A trace amount of OCT α was also detected in the bile.

Hydrolysis studies of OCT A: OCT A was converted to OCT α by the addition of pancreas, duodenum and ileum homogenate, however, little or no OCT α was detected in the liver and kidney homogenates (Table 4).

DISCUSSION

Nel and Purchase (14), van Walbeek et al. (21) and Giltier (22), using a visual analysis
reported tissue distribution and excretion of OCT A in the rat. For visual analysis, these workers used TLC and compared their findings to a standard having known amounts of OCT A as determined under UV light.

Fluorodensitometry of OCT A and labelled OCT A used in our study has made feasible acquisition of more accurate quantitative data on the tissue distribution and excretory patterns of OCT A.

Remarkable retention of OCT A in the blood was observed throughout the time course of the experimental period (Fig. 2), and it would appear that OCT A has a high affinity for blood. This hypothesis is supported in part by the results of recovery ratio when extracting OCT A from the blood (See Methods). Chu (23) has reported that OCT A binds to bovine serum albumin in vitro.

The marked decrease of OCT A in blood and tissues (Figs. 2 and 3) observed 6 hr after dosing is attributed to the temporary blockade of the elimination of OCT A through the intestinal route as a result of the catarrhal enteritis. Although the actual mechanism of acute catarrhal enteritis remains obscure, Kanisawa et al. (11) suggested that histamine and/or serotonin may be involved in the inflammation caused by OCT A.

The results in Fig. 3 indicate that the tissue distribution of OCT A coincides with the known pathology of OCT A intoxication (8). The highest concentration of OCT A per g wet weight of tissue was found in the kidney. The excretion of a high level of the radioactivity in the urine originally associated with 14C-OCT A is in good agreement with data on the pathological effect of OCT A in the urinary tract. OCT A also interacts with proteins in the kidney as has been observed in pigs (25) and chicks (26).

A relatively higher accumulation of OCT A was found in the stomach throughout the time course of the experimental period (Fig. 4) as OCT A is slowly absorbed by the stomach and small intestine.

As shown in Tables 1 and 2, the amount of OCT α detected in the feces and urine was larger than that of OCT A. The amount of OCT A excreted in the feces was much the same as that found in the urine, while the amount of OCT α excreted in the feces was twice that in the urine. This finding indicates that OCT α is not well absorbed through the intestinal tract. Nel and Purchase (14), and Giltier (22) reported that part of the OCT A was hydrolyzed to OCT α, and that OCT A was distributed in the kidney, liver, urine and feces. Van Walbeek et al. (21) demonstrated that the amount of OCT A detected in the feces and urine was greater than that of OCT α. The discrepancy between the findings of van Walbeek et al. (21) and the results described herein is attributed to different experimental conditions such as age differences and administered dose of OCT A.

With regard to the metabolism of OCT A, Yamazaki et al. (27) have demonstrated that OCT A is not hydrolyzed to OCT α by rat liver microsomal preparation in vitro. The present studies (Tables 1, 2 and 4) demonstrate that OCT A was hydrolyzed to OCT α. OCT α was also detected in the gastrointestinal tract and excreted serous fluid and mucus as determined by TLC technique. Carboxypeptidase A as well as chymotrypsin is responsible for the hydrolysis of OCT A to OCT α in vitro. (28). Carboxypeptidase A
is predominantly localized in the pancreas and small intestine (29).

In relation to the toxicity of OCT α, OCT α as well as OCT A elicited toxic effects in vitro (10), (30), but OCT α has little or no toxicity in vivo (27), (31), (32). However, Kanisawa and Suzuki (33) found that OCT α as well as OCT A produced acute catarrhal enteritis when injected into the intestinal lumen in vivo. Therefore, OCT α formed from OCT A in the small intestine may to some extent be involved in the induction of acute catarrhal enteritis in vivo.

In conclusion, the quantitative data show that OCT A is preferentially distributed to the kidneys, and the nephrotoxicity of OCT A is ascribed to its high concentration in that organ. The patterns of absorption, tissue distribution and excretion of OCT A were affected by acute catarrhal enteritis produced by OCT A and/or OCT α. The fecal and urinary excretion is mainly in the form of OCT α.

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