米穀類を汚染するカビ毒シトレオビリジンの

毒性学的研究

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Toxicological studies of citreoviridin that is a mycotoxin contaminating rice and grains

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要　旨

シトレオビリジン（CTVD）は、*Penicillium citreonigrum*、*Aspergillus terreus*、*Eupenicillium ochrosalmoneum* などが二次代謝産物として産生するカビ毒である。CTVD を産生する *P. citreonigrum* などは穀類のうち主にコメを汚染するため、コメを主食とするアジアや南米などの国々で CTVD の汚染は問題となる。CTVD に汚染されたコメが黄色くなることから、CTVD は黄変米毒の一つとしても知られており、日本国内で過去に輸入米での汚染事例が報告されている。近年においても、タイやブラジルなど熱帯性気候の地域で CTVD 産生能を有する *P. citreonigrum* の存在が報告されている。

CTVD の毒性については、1940 年に *P. citreonigrum* に汚染されたコメの粗抽出物を腹腔内投与（IP）、皮下投与（SC）又は経口投与（PO）により哺乳類や脊椎動物に投与した場合に、四肢の進行性麻痺、嘔吐、痙攣、漸次的呼吸障害などを引き起こすことがいくつかの毒性学的研究から明らかとなった。その原因カビ毒として CTVD が同定され、精製品を用いた動物実験において、神経症状の進行時間は毒素抽出物の用量が多くなるほど短くなることが報告されている。マウスに対する CTVD の LD50 は 3.6-11.8 mg/kg(皮下投与)、7.5 mg/kg(腹腔内投与)とされている。ヒトで起こる健康被害としては、衝心脚気や Keshan 病との関連も指摘されており、2006 年にブラジルで発生した衝心脚気のアウトブレイクでは、当該地域のコメから CTVD を産生する *P. citreonigrum* が検出されており、同毒素が原因物質として疑われている。

毒性学研究においてはトキシコキネティクスや体内動態の情報が欠かせないが 、CTVD の毒性実験が盛んに行われていた 1940 年代から 198 年代には微量分析が可能な分析機器等がなく、感度の低い薄層クロマトグラフィーによる蛍光分析での結果に留まっていた。そこで、本研究では CTVD のトキシコキネティクスを明らかにする目的で、解剖学的、生

理学的にヒトと類似しているブタを用いたCTVDの投与実験により検討した。また、ヒトとの比較を行うため、ヒト腸管細胞モデルであるCaco-2細胞を用いた透過実験及び代謝酵素を含む肝臓 S9 画分を用いた代謝実験を in vitro で行い、ヒトにおける CTVD のバイオアベイラビリティを推測した。本研究の概要は次のとおりである。

Ⅰ ブタにおける CTVD のトキシコキネティクス

約 10 kg のブタに CTVD 0.1 mg/kg bw を静脈投与及び経口投与した。継時的に血液を採取し、分離した血漿中の CTVD 濃度を LC-MS/MS で分析した。分析結果から解析ソフト(WinNonlin)を用いてトキシコキネティクスパラメータ(TK パラメータ)を算出し、次の結果を得た。（１）静脈投与後の CTVD の血漿中濃度は、投与後急激に減少し、最終採血時点である 48 時間後まで緩やかに減少したが 48 時間後でも血漿中から CTVD が検出された。（２）静脈投与時の TK パラメータは、小さな速度定数（Kel）（0.5 ± 0.1 $\times 10^{-1}$ h^{-1}）を示し、半減期（$T_{1/2}$）及び分布容積（Vd）はそれぞれ 16.2 ± 4.3 h、1.5 ± 0.2 L であった。（３）経口投与後の CTVD の血漿中濃度は、投与後緩やかに上昇し、15.0 ± 6.0 h（T_{max}）でピーク（Cmax：38.2 ±6.7 ng/mL）を迎えたのち、48 時間後まで緩やかに減少した。（４）経口投与時の TK パラメータは、静脈投与と同様に Kel が小さく（0.4±0.2 $\times 10^{-1}$ h^{-1}）、比較的長い $T_{1/2}$（21.4 ± 12.7 h）と大きな Vd（1.7 ± 0.3 L）を示した。（５）CTVD のブタにおけるバイオアベイラビリティは、投与後 48 時間までで 79.3 %と比較的高く、無限時間まで外挿した場合には 116.4%を示した。これらの結果から、CTVD はブタにおいて高いバイオアベイラビリティを持つことが示唆された。また、CTVD は体内からの消失が遅く、組織移行性も高いと考えられ、比較的長く体内に残留することが示唆された。

Ⅱ ヒト腸管培養細胞 Caco-2 細胞を用いた CTVD の透過係数

ヒトにおける CTVD のバイオアベイラビリティを推定するため、ヒト

腸管細胞モデルである Caco-2 細胞を用いた透過実験を実施した。Corning™ BioCoat™ Intestinal Epithelium Differentiation Environment Kit を用い、単層に培養した Caco-2 細胞の粘膜面側に CTVD（3 uM 及び 10 uM）を暴露し、37℃でインキュベートした。2 時間後の粘膜面側及び基底膜面側の培地を採取し、培地中の CTVD 濃度を LC-MS/MS を用いて測定して、経上皮電気抵抗値（TEER）及び透過係数（Papp）を求めた。その結果、（１）いずれの CTVD 濃度においても TEER に変化は認められなかった。（２）各 CTVD 濃度における Papp は、それぞれ 52.2 ± 28.3、42.6 ± 17.7 （$\times 10^{-6}$ cm/s）であり、比較的高い透過係数を示した。これらから、CTVD はブタと同様にヒトにおいても体内に吸収されやすいことが示唆された。高い透過係数は、CTVD の脂溶性の高さを反映する結果と考えられる。

Ⅲ ブタ及びヒト肝臓 S9 画分を用いた CTVD の代謝とその代謝物

ブタを用いた CTVD の in vivo 実験において、血漿中 CTVD 濃度が比較的緩やかに減少し、48 時間後においても血漿中に残留していたことから、ブタにおいては代謝が比較的遅い可能性が示唆された。そのため代謝酵素を含むブタ及びヒトの肝臓 S9 画分を用いた代謝実験により CTVD への代謝能を比較した。始めにグルコース−６−リン酸（G-6-P）及び NADP を含む溶液中に肝臓 S9 画分（0.5 mg/mL）及び CTVD（1.5 ug/mL）を加え、37℃でインキュベートした。インキュベート後 30 分、1 時間及び 4 時間後の溶液を採取し、Q-TOF で分析した。その結果、（１）CTVD の代謝物は主に水酸化-メチル化体、不飽和化体及びジヒドロキシ化体であることが推測された。（２）いずれの代謝物もヒト肝臓 S9 画分を用いたほうがブタ肝臓 S9 画分を用いるよりも有意に多く産生された。（３）CTVD 濃度がブタ肝臓 S9 画分を用いるよりもヒト肝臓 S9 画分を用いたほうが有意に低下した。これらから、CTVD は、ブタにおいて S9 による代謝が遅いことが示唆され、ヒトにおいてはブタよりも CTVD を代謝しやすいことが考えられた。

S9 画分を用いた代謝実験において、グルクロン酸抱合体が産生されなかったことから、ウリジン二リン酸グルクロン酸及びアラメチシンの存在下で CTVD と S9 画分をインキュベートして、CTVD のグルクロン酸抱合体の産生を調べた。その結果、（１）いずれの S9 画分を用いた場合でも、インキュベート後 30 分までに CTVD のグルクロン酸抱合体は産生されなかった。（２）インキュベート後 4 時間でブタ肝臓 S9 画分を用いたほうがヒト肝臓 S9 を用いるよりも有意にグルクロン酸抱合体が産生された。これらから、CTVD のグルクロン酸抱合体についてはブタのほうがヒトよりも産生されやすいことが示唆された。

本研究では、in vivo におけるブタでの CTVD のトキシコキネティクスの結果より、CTVD が体内に吸収されやすく、長い半減期と大きい分布容積を持つことが示された。これらのことから比較的長く体内に残留することを明らかにした。また、CTVD のバイオアベイラビリティはブタにおいて高いだけでなく、ヒトにおいても高い可能性を in vitro で明らかにした。一方で、CTVD の代謝については、ヒトではブタよりも早いが、グルクロン酸抱合体形成能はブタのほうが優れていたことを in vitro の観点から明らかにした。

Abstract

Citreoviridin (CTVD) is a mycotoxin that is a secondary metabolite produced by *Penicillium citreonigrum*, *Aspergillus terreus* and *Eupenicillium ochrosalmoneum*. Because *P. citreonigrum* capable of producing CTVD contaminates mainly rice, contamination with CTVD can be problem in countries of Asia and South America, where rice is a staple food. CTVD is a yellow rice toxin, and a case of its contamination in an imported rice sample was previously reported in Japan. Recently, the occurrence of *P. citreonigrum* capable of producing CTVD has been reported in areas with a tropical climate, such as Thailand and Brazil.

Regarding toxicity of CTVD, in the 1940s, several toxicological studies revealed that symptoms such as progressive paralysis, vomiting, convulsions and temporary respiratory disturbance developed when a crude extract from contaminated rice was administered to mammals and other vertebrates via intraperitoneal (IP), subcutaneous (SC) and oral (PO) routes. Furthermore, it was found that isolated

CTVD causes the same symptoms as experiments using moldy rice. Neurological symptoms progressed faster at higher doses of extracted toxin, and the median lethal dose (LD_{50}) of CTVD for mice has been reported be 3.6 to 11.8 mg/kg (SC) and 7.5 mg/kg (IP). As a human adverse effects, CTVD has been considered to be related to Shoshin-kakke (acute cardiac beriberi) and Keshan disease which adversely affects the heart. In 2006, an outbreak of Shoshin-kakke occurred in Brazil, and the presence of rice contaminated with *P. citreonigrum* capable of producing CTVD was reported in the outbreak area.

An understanding of toxicokinetics is essential when performing a toxicological study. However, from the 1940s to the 1980s, when toxicological studies of CTVD were performed actively, no high-resolution analytical methods had yet been developed, and the toxicokinetics of CTVD were determined based solely on the results of a fluorescence analysis using thin layer chromatography.

The present study was conducted with the following three experiments: (1) the

toxicokinetics of CTVD were investigated by administering CTVD to swine, which are similar to humans both anatomically and physiologically; (2) the permeability of CTVD was investigated using Caco-2 cells, which are a model of human intestinal cells; and (3) a CTVD metabolic study was conducted using the swine or human hepatic S9 fraction containing metabolic enzymes.

The bioavailability of CTVD to humans was estimated, while in addition the toxicokinetics of CTVD in vitro to humans was compared with that to swine on studies (2) and (3).

I. Toxicokinetics of CTVD in swine

CTVD (0.1 mg/kg body weight) was administered to swine (weighing about 10 kg) intravenously (IV) and PO. The CTVD concentration of plasma samples was determined via liquid chromatography with tandem mass spectrometry (LC-MS/MS). The toxicokinetics parameters (TK) were analyzed, and the following results were obtained: (1) the CTVD concentration in plasma decreased quickly shortly after IV

administration and then continued to decrease moderately until 48 h (final sampling point); however, some amount of CTVD remained in plasma even at 48 h after administration. (2) The rate constant (Kel) after IV administration was 0.5 ± 0.1 × 10^{-1} h^{-1}. The half-life ($T_{1/2}$) and volume of distribution (Vd) were 16.2 ± 4.3 h and 1.5 ± 0.2 L, respectively. (3) The CTVD concentration in plasma in PO gently increased after administration and reached the maximum concentration (C_{max}: 38.2 ± 6.7 ug/mL) at 15.0 ± 6.0 h (T_{max}), after which it moderately decreased until 48 h. (4) The Kel after PO administration was small (0.4 ± 0.2 × 10^{-1} h^{-1}, similar to that in IV). The $T_{1/2}$ and Vd were 21.4 ± 12.7 h and 1.7 ± 0.3 L, respectively. (5) The bioavailability of CTVD was 79.3% from 0 to 48 h and 116.4% on extrapolating from 48 h to infinity. These results suggested that CTVD had a high bioavailability in swine. In addition, CTVD was eliminated slowly from the body and was easily distributed to tissues. These findings suggested that CTVD remained in the body for a relatively long time after administration.

II. Permeability of CTVD for Caco-2 cells, a human intestinal cell model

Because CTVD had a relatively high bioavailability in swine, a permeability study was performed using Caco-2 cells, which are a human intestinal cell model. CTVD (3 μM and 10 μM) was treated to the apical side (AP) of Caco-2 cells cultured in a monolayer using a Corning™ BioCoat™ Intestinal Epithelium Differentiation Environment Kit (Corning, NY, USA) and then incubated at 37 °C. After 2 h, the culture medium was sampled from the AP and basolateral side (BL). The CTVD concentration was determined using LC-MS/MS, and then the transepithelial electrical resistance (TEER) and permeability coefficient (PaPP) were estimated. The results of this study were as follows: (1) The TEER was unchanged at each concentration of CTVD. (2) The PaPP values at each CTVD concentration of 3 μM and 10 μM were 52.2 ±28.3 and 42.6 ± 17.7 ($\times 10^{-6}$ cm/s), respectively, indicating a relatively high permeability. These findings suggested that CTVD could be easily absorbed by the human intestine, similar to swine, which reflected a high lipid

solubility of CTVD.

III. CTVD metabolism and its metabolites as assessed using swine and human hepatic S9 fractions

Our in vivo study showed that the plasma CTVD concentration decreased gently, and CTVD was still detected even at 48 h after administration. Given these results, the CTVD metabolism was next investigated using swine and human hepatic S9 fractions containing metabolic enzymes. CTVD was incubated at 37 °C with hepatic S9 fraction in solution containing glucose-6-phosphate (G6P) and NADP. The solution was sampled at 30 min, 1 and 4 h after incubation, and then the concentrations of CTVD and its metabolites were determined using a quadrupole time-of-flight mass spectrometer (Q-TOF). The findings were as follows: (1) The CTVD metabolites were estimated to be hydroxylation-methylation, desaturation and dihydoroxylation derivatives. (2) Each metabolite was produced in greater quantities using the human hepatic S9 fraction than the swine hepatic S9 fraction. (3) The

CTVD concentration was significantly lower with the human hepatic S9 fraction than with the swine hepatic S9 fraction. These results suggested that CTVD was slowly metabolized in swine, which may have been the cause of the slow elimination rate noted in the swine in the in vivo study. In addition, humans seemed to more readily metabolize CTVD than swine.

Because CTVD glucuronide was unable to be detected in the metabolic study using S9 fractions in the presence of G6P and NADP, CTVD was incubated with an S9 fraction in the presence of UDP-glucuronic acid and alamethicin to examine CTVD glucuronidation ability. The following results were obtained: (1) CTVD glucuronide had not been produced by 30 min after incubation, regardless of the fraction used. (2) The levels of CTVD glucuronide at 4 h after incubation were significantly greater with the swine hepatic S9 fraction than with the human hepatic S9 fraction. These findings suggested that CTVD glucuronide was more readily produced in swine than in humans.

The present studies showed that CTVD is easily absorbed by the body and has a long $T_{1/2}$ and large Vd, indicating that CTVD persists for a relatively long time in the body. In addition, the bioavailability of CTVD appeared to be high in both humans and swine. Regarding CTVD metabolism, humans were able to metabolize CTVD earlier than swine according to in vitro findings, while the glucuronidation ability for CTVD was better in swine than in human from an in vitro perspective.

toxins

MDPI

Article

The In Vivo and In Vitro Toxicokinetics of Citreoviridin Extracted from *Penicillium citreonigrum*

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Abstract: Citreoviridin (CTVD), a mycotoxin called yellow rice toxin, is reported to be related to acute cardiac beriberi; however, its toxicokinetics remain unclear. The present study elucidated the toxicokinetics through in vivo experiments in swine and predicted the human toxicokinetics by comparing the findings to those from in vitro experiments. In vivo experiments revealed the high bioavailability of CTVD (116.4%) in swine. An intestinal permeability study using Caco-2 cells to estimate the toxicokinetics in humans showed that CTVD has a high permeability coefficient. When CTVD was incubated with hepatic S9 fraction from swine and humans, hydroxylation and methylation, desaturation, and dihydroxylation derivatives were produced as the predominant metabolites. The levels of these products produced using human S9 were higher than those obtained swine S9, while CTVD glucuronide was produced slowly in human S9 in comparison to swine S9. Furthermore, the elimination of CTVD by human S9 was significantly more rapid in comparison to that by swine S9. These results suggest that CTVD is easily absorbed in swine and that it remains in the body where it is slowly metabolized. In contrast, the absorption of CTVD in humans would be the same as that in swine, although its elimination would be faster.

Keywords: citreoviridin; toxicokinetics; bioavailability; swine; Caco-2; S9

Key Contribution: CTVD bioavailability was high in swine in vivo; and was predicted to be high in humans based on an in vitro Caco-2 permeability study. Human S9 metabolized CTVD to hydroxylation, methylation, desaturation, and dihydroxylation metabolites and the activity was higher in comparison to swine. The glucuronidation activity of swine S9 was higher than that of human S9. The elimination of CTVD obtained by human S9 was faster than that by swine S9 in vitro.

1. Introduction

Citreoviridin (CTVD) (Figure 1) is a mycotoxin produced as a secondary metabolite of *Penicillium citreonigrum*, *Aspergillus terreus*, and *Eupenicillium ochrosalmoneum* [1]. CTVD has previously been detected in food including rice in various areas [2–6]. Contamination with CTVD, well-known as yellow rice toxin, often occurs due to inappropriate management after harvest [7]. The toxicity of CTVD is mainly accompanied by ascending paralysis, central nervous system disturbance and respiratory arrest, and fatal adverse effects may occur [8]. Because CTVD is mainly found as a contaminant in rice,

these properties of CTVD can be a serious problem in countries in which people consume rice as a staple food such as Thailand, Brazil, and Japan.

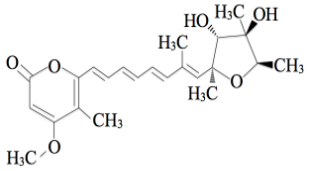

Figure 1. The chemical structure of citreoviridin (CTVD).

In a 2006 outbreak of beriberi in Brazil, 1207 people were diagnosed with beriberi, and 40 patients died [9]. Since *P. citreonigrum* and CTVD were detected in rice samples, CTVD was the suspected cause of the beriberi outbreak in that area [10].

Regarding the relationship between CTVD and beriberi, Uraguchi et al. reported, based on the results of an epidemiological investigation, the possibility of acute cardiac beriberi, which induces neurological symptoms and heart failure [11]. Ueno et al. reported that CTVD produced neurological symptoms similar to acute cardiac beriberi in humans based on experiments in mice and rats [12]. This disease has been caused by the consumption of moldy rice in areas in which rice preservation was inadequate. Studies on CTVD toxicity were performed using animal experiments from the 1940s to 1980s. These studies revealed that CTVD causes fatal adverse effects, with symptoms by ascending paralysis, disturbance of the central nervous system, and respiratory arrest [8]. The lethal dose 50% (LD_{50}) of CTVD in mice was reported to be 3.6–11.8 mg/kg subcutaneously and 7.5 mg/kg intraperitoneally [13,14]. When crude extract from yellow rice was administered to several mammals subcutaneously, intraperitoneally, and per os, the abovementioned typical neurological symptoms were observed [15]. In addition, the development of these symptoms in rats occurred earlier when the dose was increased [16]. Chronic exposure to CTVD has also been reported as a possible trigger of Keshan disease (an endemic disease in which patients present cardiomyopathy) [17]. Sakai et al. focused on the chronic toxicity at low doses, and reported that rats died when 1/300 of the oral LD_{50} dose was administered subcutaneously every day for a six-month period [16]. This suggested that CTVD might accumulate in the body and have cumulative effects. Furthermore, the distribution of CTVD in the liver, kidney, heart, and brain, as determined by a photometric analysis, showed that CTVD remained in the liver, kidney, and heart of rats even at 52 h after its subcutaneous administration (SC) [12]. Although these results show the possibility that CTVD may easily remain in the body, the kinetics parameters, including oral bioavailability, have rarely been described.

The present study considered the cumulative properties of CTVD by elucidating the toxicokinetics and bioavailability of CTVD in vivo. Moreover, to estimate the bioavailability of CTVD in humans, the metabolic efficiency of CTVD in vitro was compared by an S9 metabolic study, and a permeability study was conducted using Caco-2 cells as a human intestinal cell model. First, the kinetics of CTVD in plasma were investigated by administering CTVD intravenously and orally to swine in an in vivo study. Subsequently, the intestinal permeability of CTVD was evaluated using Caco-2 cells and the metabolism of CTVD and the production of its main metabolites were investigated using S9 fractions obtained from swine and humans. The parameters thus obtained may contribute to improving the knowledge on the risk of CTVD.

2. Results

2.1. The Toxicokinetics of CTVD in Swine

None of the swine that received CTVD intravenously or orally showed adverse clinical signs. The plasma CTVD concentration profile after intravenous administration (IV) is shown in Figure 2a. The rate constant (Kel) was small ($0.5 \pm 0.1 \times 10^{-1}$ h^{-1}) (Table 1). The mean half-life of CTVD was

16.2 ± 4.3 h, which was relatively long. The volume of distribution (Vd) was greater than the total body water (1.5 ± 0.2 L), and the mean residence time (MRT) of CTVD was estimated to be 14.6 ± 1.2 h. To calculate the bioavailability of CTVD based on the different estimated parameters, the area under the curve (AUC) was determined to be 1512.9 ± 331.7 h·ng/mL, with extrapolation to infinity.

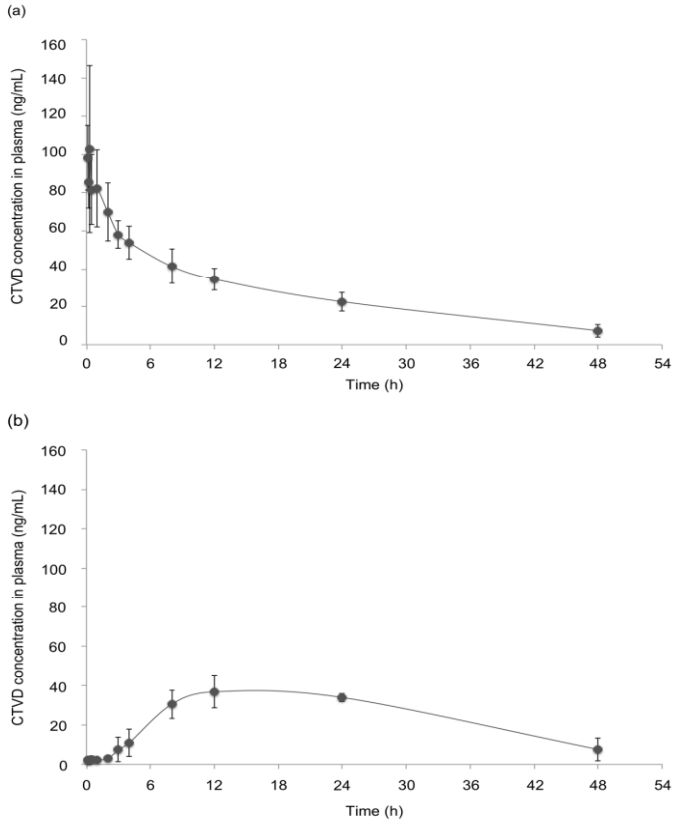

Figure 2. The CTVD concentration–time profiles in the plasma of swine after IV and oral administration (PO). CTVD (0.1 mg/kg·BW) was administered intravenously (**a**) and orally (**b**). Control plasma was obtained on the day before administration. Values are presented as the mean ± standard deviation (SD). $n = 4$ in both groups.

Table 1. The toxicokinetic parameters in swine that received CTVD intravenously and orally.

	BW (kg)	Kel $\times 10^{-1}$ (h^{-1})	$T_{1/2}$ (h)	Vd or Vd/F (L)	MRT (h)	AUC_t (h·ng/mL)	AUC∞ (h·ng/mL)	C_{max} (ng/mL)	T_{max} (h)	F_t (%)	F (%)
IV	9.4 ± 1.3	0.5 ± 0.1	16.2 ± 4.3	1.5 ± 0.2	14.6 ± 1.2	1322.2 ± 224.4	1512.9 ± 331.7	-	-	-	-
PO	10.7 ± 1.3	0.4 ± 0.2	21.4 ± 12.7	1.7 ± 0.3	19.6 ± 4.0	1048.2 ± 180.8	1761.1 ± 813.5	38.2 ± 6.7	15.0 ± 6.0	79.3	116.4

BW, body weight; C_{max}, maximum plasma CTVD level; T_{max}, time of maximum plasma CTVD concentration; Kel, the rate constants; $T_{1/2}$, biological half-life of the elimination; Vd or Vd/F, apparent volume of distribution in IV or PO; MRT, mean residence time; AUCt, area under the curve from the curve 0 to the last quantifiable concentration; AUC∞, area under the curve from the curve 0 to infinity; F_t, bioavailability calculated using mean AUC_t after PO and IV; F, bioavailability calculated using mean AUC∞ after PO and IV. With the exception of F_t and F, all values are presented as the mean ± SD.

The CTVD concentration profile in plasma after PO administration is shown in Figure 2b. The peak plasma concentration (C_{max}, 38.2 ± 6.7 ng/mL) was observed at 15.0 ± 6.0 h (T_{max}) after PO (Table 1). The Kel was 0.4 ± 0.2 $\times 10^{-1}$ h^{-1}, which was low and similar to the value after IV administration.

The $T_{1/2}$ was approximately one day (21.4 ± 12.7 h), and the Vd/F was relatively large (1.7 ± 0.3 L). The MRT obtained from those values was relatively long (19.6 ± 4.0 h) (Table 1), suggesting that CTVD persisted in the bodies of swine. The AUC extrapolated to infinity was 1761.1 ± 813.5 h·ng/mL. The estimated bioavailabilities in swine from AUC_t and AUC∞ was 79.3% and 116.4%, respectively.

2.2. Permeability Study Using Caco-2 Cells

The results of the administration study showed that CTVD had high bioavailability in swine. In order to compare the intestinal permeability of CTVD in humans with the bioavailability of CTVD in swine, the permeability was investigated using Caco-2 cells. The Caco-2 cell model is an in vitro model used to evaluate intestinal permeability and the influence of chemical compounds on the intestinal barrier function in humans.

The apparent permeability coefficient (Papp) estimated from a Caco-2 permeability assay has been reported to be well correlated with the human in vivo absorption data for many agents [18,19]. The transepithelial electrical resistance (TEER) value is generally accepted to reflect the integrity of the tight junction dynamics in Caco-2 cells [20]; thus, the TEER was measured at 1 and 2 h after exposure to 3 and 10 μmol/L of CTVD. Our results showed no marked change in the TEER over time at any concentration (data not shown). The rate of CTVD transport from the apical (AP) side to the basolateral (BL) side was calculated based on the concentration of CTVD in the BL compartment. The Papp was calculated as described in a previous paper [21]. The Papp after 2 h of incubation with 3 and 10 μmol/L CTVD was 52.2×10^{-6} and 42.6×10^{-6} (cm/s), respectively, in the AP-BL direction (Table 2). These findings indicated that human intestinal cells were highly permeable to CTVD in vitro.

Table 2. The Papp at each concentration of CTVD.

Parameter	3 μmol/L	10 μmol/L
Papp ($\times 10^{-6}$ cm/s)	52.2 ± 28.3	42.6 ± 17.7

Papp, apparent permeability coefficient of Caco-2 cells treated with 3 and 10 μmol/L CTVD in an AP chamber for 2 h. Values are presented as the mean ± SD.

2.3. CTVD Elimination and the CTVD Metabolite Profile Following Incubation with S9 Fraction In Vitro

The fact that it took more than 40 h to eliminate CTVD in plasma after IV or PO administration in swine in vivo experiments suggested that CTVD was poorly metabolized in the liver. Thus, to confirm the metabolic activity of CTVD and the metabolites of CTVD produced in the liver, the residual CTVD concentration was measured after incubation in vitro with hepatic S9 fraction from swine, and the profile of the CTVD metabolites produced was determined using Q-TOF. These data obtained from the swine S9 fraction were subsequently compared to those obtained using human S9 fraction.

The elimination of CTVD as well as the metabolites produced when CTVD was incubated with S9 fraction supplemented with NADP was investigated. NADP is a coenzyme of dehydrogenase that is often used in metabolism assays with S9 [22,23]. Incubation was conducted according to the method of Wu et al. [24]. As a result, hydroxylation and methylation, desaturation, and dihydroxylation derivatives were detected as the main metabolites of CTVD. The extracted ion chromatogram (EIC) and accurate mass spectra of these metabolites are shown in Figure A1 (Appendix A). These detected metabolites were confirmed from monoisotopic mass and mass accuracy (Table A1). Although the concentration of CTVD incubated with human hepatic S9 fraction decreased as the duration of incubation increased, the concentration of CTVD incubated with swine hepatic S9 fraction was almost unchanged from 30 min of incubation to 240 min of incubation (Figure 3a). Furthermore, the concentration of CTVD after 240 min of incubation with human hepatic S9 fraction was significantly lower than that after incubation with swine hepatic S9.

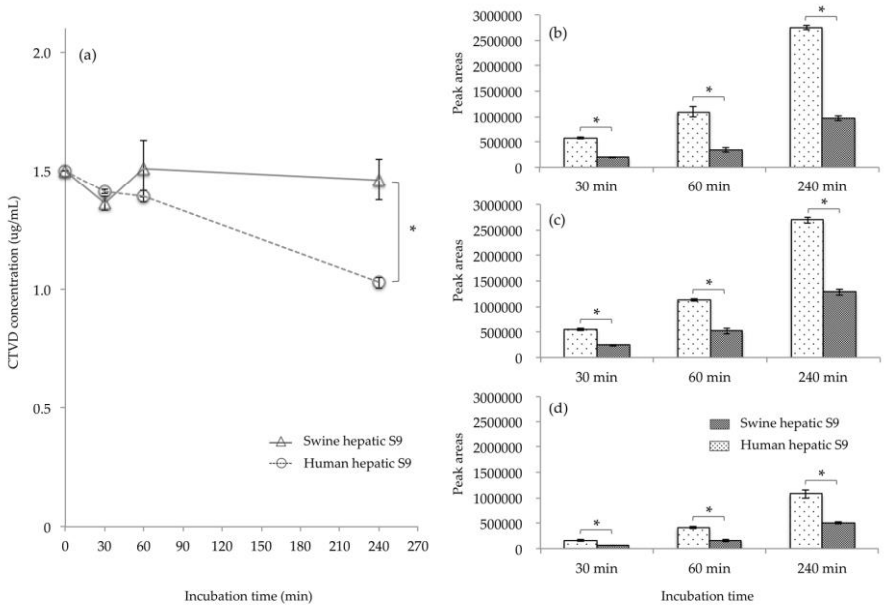

Figure 3. The comparison of the CTVD concentration and the main metabolites in humans and swine, obtained by incubating of CTVD with hepatic S9 fractions of humans and swine. CTVD (1.5 μg/mL) was incubated with the hepatic S9 fractions of humans and swine supplemented with NADP as a coenzyme. The CTVD concentrations at 30, 60, and 240 min after the start of incubation are described (**a**). The main metabolites produced in humans and swine are shown in (**b–d**). The metabolites in humans and swine at each time-point were compared based on the mean peak area of each metabolite. (**b–d**) show the mean peak areas of hydroxylation and methylation, desaturation, and dihydroxylation derivatives, respectively, following incubation with human or swine hepatic S9, respectively. Values are presented as the mean ± SD. Asterisks indicate a statistically significant difference ($p < 0.05$).

Since the hydroxide of CTVD did not show a quantitative change over time from the metabolic reaction of S9, products other than those derived from the control group due to the metabolic reaction with S9 were considered to be metabolites of CTVD. The main metabolite forms of CTVD were found to have been produced by hydroxylation and methylation, desaturation, and dihydroxylation. The main metabolites could not be quantified because standard substances of the metabolites detected were not commercially available. Thus, the metabolites in humans and swine at each time-point were compared based on the mean area of each metabolite. The metabolite peak area after incubation with human hepatic S9 fraction was two to three times higher than that after incubation with the swine hepatic S9 fraction for all metabolites (Figure 3b–d). These results indicate that human hepatic S9 fraction has a greater ability to metabolize CTVD than swine hepatic S9 fraction.

2.4. CTVD Elimination and the CTVD Glucuronide Profile Following Incubation with S9 In Vitro

Glucuronide is a well-known metabolite produced from the detoxification of mycotoxins. However, after incubation with S9 fraction and NADP as a coenzyme, no glucuronide was recognized in our swine or human metabolite models. Moreover, it is reported that swine have low sulfate conjugation ability; this has been reported to be alternated by processes such as glucuronidation [25]. For this reason, the glucuronidation of CTVD in the presence of uridine-5′-diphosphoglucuronic acid trisodium salt (UDPGA) was examined in order to compare the detoxification ability between swine and humans. UDPGA is a cosubstrate used in the glucuronidation reaction [26]. Swine and human hepatic S9 were used for incubation, and CTVD glucuronide was observed at 30, 60, and 240 min after incubation. The EIC and accurate mass spectrum of CTVD glucuronide are shown in Figure A2. Of note, with

swine hepatic S9, CTVD glucuronide was detected at 60 min and the amount increased over time until 240 min, while CTVD glucuronide was not detected at 30, 60, or 240 min with human hepatic S9 (Figure 4).

Because the standard substance of CTVD glucuronide was not commercially available, the same method that was used for the comparison of S9 fractions supplemented with NADP was applied. The mean area of CTVD glucuronide after incubation with swine hepatic S9 fraction for 240 min was approximately twice that after incubation with human hepatic S9 fraction for 240 min (Figure 4).

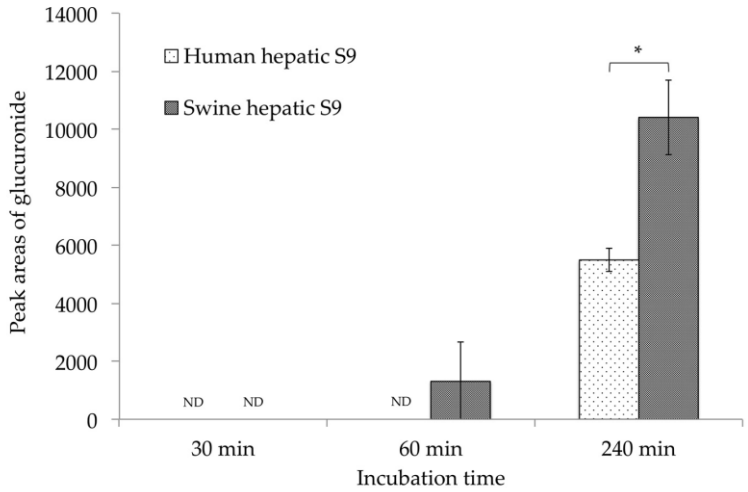

Figure 4. A comparison of the CTVD glucuronidation during incubation with human and swine S9 fractions supplemented with UDPGA. CTVD (1.5 μg/mL) was incubated with human and swine hepatic S9 fractions supplemented with UDPGA as a coenzyme. The amount of CTVD glucuronide at 30, 60, and 240 min was measured by Q-TOF. The metabolites in humans and swine at each time-point were compared based on the mean area of each metabolite. Values are presented as the mean ± SD. Asterisks indicate a statistically significant difference ($p < 0.05$). ND, not detected.

3. Discussion

Swine are known to share some physiological and anatomic similarities with humans, including food habits. The toxicokinetics of CTVD were therefore investigated in swine for extrapolation to humans. The bioavailability of mycotoxins has been reported for several major compounds. Deoxynivalenol (48–109.8% in swine) [27–29] and ochratoxin A (65.7% in swine) [30] have relatively high bioavailability, and the bioavailability of zearalenone in swine is reported to be 80–85% [31]. The result of the present study was over 100% by calculated from AUC∞. This was possible to have been overestimated, because the extrapolation estimate (from 48 h to infinity) in PO administration had 68% of AUC_t. However, the bioavailability estimated from AUC_t (from 0 to 48 h) was 79.3%, then it was suggested that CTVD was also a mycotoxin with similarly high bioavailability to these compounds (Table 1). Generally, lipophilic substances appear to be easily absorbed by the intestine through passive transport. Aflatoxin and zearalenone are lipophilic and low-molecular mass molecules that are said to be transported through passive diffusion [32]. CTVD is a similarly lipophilic and low-molecular mycotoxin with a low-molecular mass; thus, it is considered to be absorbed by passive transport. Lipophilic compounds access the systemic circulation through the intestinal lymphatic system by which these compounds avoid the first pass effect [33]. Thus, these properties of CTVD may be one reason for its high bioavailability.

In the permeability experiment using Caco-2 cells, CTVD showed a high Papp (Table 2) despite having no marked effect on the TEER. The Papp of CTVD was similar to that of propranolol as a model

of lipophilic drug [34]. Furthermore, the Papp was higher than the Papp values of deoxynivalenol (0.19 ± 0.02 [× 10^{-6} cm/min]) [35] and zearalenone (10.4 ± 4.7 [× 10^{-6} cm/s]) [36], although it was lower than that of aflatoxin M1 (105.10 ± 7.98 [×10^{-6} cm/s]) [37]. The permeability coefficient from the Caco-2 cell assay has been shown to be correlated with the bioavailability and intestinal absorbency, following a sigmoidal curve (wherein a substance with a high permeability coefficient has high absorbency) [18,19]. Considering that the results of our in vivo study using swine indicated the relatively higher bioavailability of CTVD, the result of the Caco-2 study suggested that the bioavailability of CTVD in humans would be similarly high to that in swine.

The plasma CTVD concentration showed almost no increase until 3 h after PO to swine (Figure 2b). The dwell time of digesta in the stomach of swine has been reported to range from one to three hours [38]. As CTVD was administered with feed in this study, CTVD may have been retained with digesta in the stomach. Some mycotoxins have been reported to be absorbed from the stomach [39,40]. However, in the present study, a marked increase in the plasma CTVD concentration was noted from three hours after its administration; thus, CTVD may be poorly absorbed from the stomach.

In the in vivo study, the elimination of CTVD from the body of swine appeared to be quite slow, the Vd of CTVD in swine was greater than 1 L (Table 1). Generally, drugs with a Vd exceeding 1 L are considered to be widely distributed to the body tissue [41]. Ueno et al. [12] performed an in vivo study on the distribution and elimination of CTVD, which supported our results. In their study [12], they noted that after the SC of extracted CTVD, CTVD was rapidly distributed from the site of administration to the main organs, including the liver, kidney, and heart. They also found that the concentration of CTVD was highest in the liver after 8 h, and that <1% of the total administered dose could still be detected in the liver, even after 52 h. Thus, from the results of the present study, CTVD was suggested to be widely distributed to the body tissue. Regarding other mycotoxins, ochratoxin A (84.5 h in swine, 840 h in monkey) [29,42] and aflatoxin B1 (91.8 h in rat) [43] are reported to have long elimination half-lives. In contrast, the elimination half-lives of fumonisin B1 (182 min in swine) and deoxynivalenol (7.2–15.2 h in swine) are reported to be short [44]. In this respect, CTVD is a mycotoxin with a relatively long half-life (Table 1). Hou et al. proposed that CTVD bound plasma albumin [45]. Ochratoxin A is generally accepted to bind plasma albumin [46,47], which may explain why CTVD showed a long elimination half-life. In addition, Sakai et al. reported that extract from yellow rice had a lethal effect, even when exposed rats were given daily doses of 1/300 of LD_{50} PO for 16 months [16]. In the present study, the toxicokinetics of CTVD in swine showed that CTVD had high bioavailability and persisted in the body for a relatively long period of time. This suggested that CTVD might accumulate in the body with chronic exposure, and this result was considered to reflect the above report describing the adverse effects of chronic exposure.

To estimate the bioavailability in humans, the metabolites in human hepatic S9 fraction were examined and compared to those produced by swine S9 fraction in an in vitro experiment. Regarding the metabolites present in the S9 fraction of humans, the main metabolites—including hydroxylation and methylation, desaturation, and dihydroxylation derivatives—were the same as those in the swine S9 fraction (Figure 3). Marked differences were noted in the metabolite-producing ability of the hepatic S9 fraction; the metabolization when human S9 fraction was used was higher than that when swine S9 fraction was used. Although glucuronide was detected among the metabolites produced by the hepatic S9 fractions of both species when supplemented with UDPGA, the glucuronidation of CTVD in humans was shown to be slower in comparison to in swine (Figure 4). Interspecies differences in the hepatic glucuronidation of deoxynivalenol have been reported [48]. Moreover, swine have no sulfate conjugation ability (or lower ability in comparison to other species); thus, it has been shown that sulfatic conjugation in swine occurs via pathways other than phase II pathways [25]. Furthermore, in the metabolism of CTVD, glucuronidation might compensate for sulfatic conjugation in swine due to their low sulfate conjugation ability, and this was considered to be a factor that caused the increased production of glucuronide from CTVD in pigs in comparison to humans. On the other hand, because CTVD was more metabolized in humans than in pigs, it was suggested that in humans, CTVD may

be metabolized by phase II pathways other than glucuronidation. Further investigations should be performed to test this hypothesis. Overall, the results of our in vitro study using S9 fraction suggested that the metabolism of CTVD in the human liver would be faster than that in the swine liver (Figure 3).

One limitation associated with the present study is that frequent blood drawing within the first hour after administration was performed under anesthesia, due to considerations for the animals' welfare, and that the effect in relation to absorption and metabolism was unclear. However, the results of the present study are important in that the toxicokinetic parameters in the in vivo and in vitro experiments clearly demonstrated that CTVD can easily remain in the body. This is in line with the results from previous animal experiments. In the future, although it will be necessary to accumulate further data, this research may provide useful information for evaluating the risk associated with the administration of CTVD.

Although previous animal experiments demonstrated that CTVD remains in the body for a long time, in this study we performed metric analyses to reveal the toxicokinetics for the first time. The results indicated that CTVD has high bioavailability in swine and that it persisted in the body for a relatively long time. Thus, CTVD may bring about adverse effects due to accumulation as a result of chronic exposure. In addition, the comparison of the in vitro findings in humans and swine suggested that the bioavailability of CTVD in humans was similarly high to that in swine, although CTVD appears to be metabolized more quickly in humans than in swine.

4. Materials and Methods

4.1. Reagents

CTVD (Figure 1) (purity: 88.8%) was extracted from *P. citreonigrum* isolated by Shiratori et al. [6] with reference to the method of da Rocha et al. [49]. Regarding the approximately 12% impurities, most were hydroxides of CTVD. These hydroxides did not change quantitatively during the reaction of S9 (data not shown). The Caco-2 cell lines were provided by the Division of Pharmacognosy, Phytochemistry, and Narcotics of the National Institute of Health Sciences (Kanagawa, Japan). Human and swine hepatic S9 fractions were purchased from Sekisui XenoTech, LLC. (Kansas City, KS, USA). NADP, Glucose-6-Phosphate, Hank's balanced salt solution (HBSS) and HEPES were purchased from Sigma-Aldrich (St. Louis, MO, USA). UDPGA was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Alamethicin was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). Inactivated fetal bovine serum was obtained from Biowest (Nuaillé, France). Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Invitrogen Japan (Tokyo, Japan). Nonessential amino acids were obtained from MP Bio Science (Derbyshire, UK). The Corning™ BioCoat™ Intestinal Epithelium Differentiation Environment Kit was purchased from Corning (NY, USA). Medetomidine hydrochloride and butorphanol tartrate were obtained from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Midazolam was obtained from Astellas Pharma Inc. (Tokyo, Japan). Other reagents were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan).

4.2. Administration Study

4.2.1. Animals and Diets

Swine (barrows; Landrace × Large White × Duroc) were obtained from CIMCO Co., Ltd. (Tokyo, Japan). They were housed in individual cages (0.88 m wide, 1.3 m deep), with ad libitum access to water, and were fed a commercial formula feed in quantities of 1.5–2% of their body weight (BW) daily. All protocols were approved by the Animal experiment ethics committee of Azabu University (Approval number: 170829-1).

4.2.2. Administration and Blood Sampling

CTVD stock solution was prepared by dissolving CTVD in acetonitrile to a concentration of 10 mg/mL. The required amount of CTVD was moved from the stock solution into a tube and dried with nitrogen. The dried CTVD was then redissolved in ethanol–saline (ratio, 1:4) and was used as a test solution. Following three days of acclimatization, administration studies were carried out. CTVD (0.1 mg/kg·BW) was intravenously administered to swine (n = 4) via the auricular vein. For PO administration (n = 4), 10 mg/mL of CTVD–ethanol solution and a small amount of water were added to feed (10 g), for a dose of 0.1 mg/kg·BW. This was then fashioned into a sphere and fed to the animals at the time of feeding. It was visually confirmed that the animals had eaten the CTVD-contaminated feed.

Blood was sampled from the jugular vein at 0 min (before administration), and 5, 10, 20, and 30 min and 1, 2, 3, 4, 8, 24, and 48 h after administration. Blood samples were placed into heparinized tubes and stored on ice until centrifugation. After centrifugation (1919× g, 10 min), the plasma was temporarily stored at −80 °C. Plasma samples were prepared according to the method of Devreese et al. [50]. A three-fold volume of acetonitrile was mixed with the plasma samples, which were then centrifuged again (8500× g, 4 °C 10 min) after mixing with a vortex mixer for 15 s. The supernatants were transferred into amber screw-top vials and dried under nitrogen gas. The samples were stored at −30 °C until the analysis.

In order to frequently sample blood via a cervical part of swine in a short period of time (up to 1 h after administration), taking animal welfare into account, CTVD was administered intravenously and orally, followed by the immediate administration of 0.1 mg/kg of mixed anesthetics (medetomidine hydrochloride:midazolam:butorphanol tartrate = 3:2:2) via intramuscular injection. Therefore, blood samplings from 5 min to 1 h after CTDV administration were conducted under anesthesia. Each animal was awake at approximately 1 h after anesthetization, blood samplings from 2 to 48 h after CTDV administration were conducted under awakening.

4.2.3. Toxicokinetic Analyses

The toxoicokinetics were analyzed using the Phoenix WinNonlin 6.4 software program (Certara, St. Louis, MO, USA). The bioavailability was determined using the following equation by calculating the AUC of the IV and PO data, which were determined with extrapolation to infinity.

$$\mathrm{F} = \frac{AUC_{\,PO} / Dose_{\,PO}}{AUC_{\,IV} / Dose_{\,IV}} \times 100$$

where F is the bioavailability of CTVD. $AUC_{\,PO}$ or $_{IV}$ represents the mean area under the curve after PO or IV administration. $Dose_{\,PO}$ or $_{IV}$ represent the actual dose by PO or IV administration.

4.3. Permeability Study Using Caco-2 Cells

Cell culture and a permeability study were carried out by the method of Kadota et al. [21]. CTVD solutions (3 and 10 mmol/mL) were prepared by dissolving dried CTVD in DMSO. The permeability study was carried out using a Corning™ BioCoat™ Intestinal Epithelium Differentiation Environment Kit (Corning, NY, USA). Cell incubation and induction of differentiation were performed in accordance with the protocol of the kit. CTVD solutions were added to Enterocyte Differentiation Medium (EDM) containing 0.08% MITO + serum extender, with CTVD at concentrations of 3 and 10 μmol/L.

EDM containing CTVD was exposed to Caco-2 cells from the apical (AP) side. The TEER of the AP and BL sides was measured at 0 (before exposure), 1 and 2 h (after exposure) using a Millicell ERS device (Millipore, Molsheim, France). To determine the CTVD concentration at the AP and BL sides, transport buffer was collected from both sides. After transferring 400 μL of collected buffer (per side) to a microtube, a three-fold volume of acetonitrile was added. Samples were mixed with a vortex mixed, followed by centrifugation at 8500× g for 10 min at 4 °C. The supernatant was then transferred

to an amber vial and dried with nitrogen gas. Samples were stored at −30 °C until an analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

4.4. Production of CTVD Metabolites by Incubating with S9 Fractions

The incubation of S9 supplemented with NADP was carried out with reference to a previous report [24]. CTVD stock solution (25 µL) was transferred into a microtube and dried with nitrogen. CTVD solution (250 µg/mL) was prepared by redissolving dried CTVD with 1 mL of DMSO. CTVD additive solution (150 µg/mL) was prepared by mixing 600 µL of CTVD solution (250 µg/mL) and 400 µL of a base buffer. The total volume of the test solution was 500 µL. The final concentrations of each factor in the test solution were as follows: $MgCl_2$ (5 mmol/L), Glucose-6-phosphate (5 mmol/L) and NADP (0.5 mmol/L). The concentrations of S9 and CTVD in the test solution were 0.5 mg/mL and 1.5 µg/mL, respectively. After adding CTVD, the test solution was incubated in a warm bath at 37 °C for 30, 60, or 240 min. The reaction of the test solution was terminated by adding the same amount (500 µL) of acetonitrile. Each sample was mixed in a vortex mixer at 30 s, followed by centrifugation at 6000× *g* for 10 min at 4 °C. The supernatant was transferred to an amber vial and dried with nitrogen gas. Dried samples were stored at −30 °C until the analysis.

S9 was incubated with UDPGA as follows: first, a mixture (S9 (final concentration, 0.5 mg/mL), Tris-HCl buffer (pH 7.4; final concentration, 50 mmol/L), $MgCl_2$ (final concentration, 0.5 mg/mL), alamethicin (final concentration, 0.25 µg/mL) and CTVD (final concentration, 1.5 µg/mL)) was pre-incubated at 37 °C for 5 min. The total volume was then brought to 1 mL by adding UDPGA (final concentration, 3 mmol/L), and incubation was started at 37 °C. A 100 µL aliquot of the sample was collected from each mixture at 30, 60, and 240 min from the start of incubation. An equal amount of acetonitrile was then added to terminate the reaction. After centrifugation at 9000× *g* for 5 min at 4 °C, the supernatant was dried with nitrogen gas. Samples were stored until use at −30 °C.

4.5. Quantification of CTVD and Detection of Metabolites

Dried samples from the administration and permeability studies were redissolved in methanol for the analysis. The quantification of CTVD in samples was conducted under the following analytical conditions: LC was performed using an Agilent 1290 Infinity LC System (Agilent Technology Ltd., Santa Clara, CA, USA), and separation was performed using a ZORBAX Eclipse plus C18 (100 mm, 2.1 mm, 1.8 µm; Agilent Technology Ltd.). The mobile phases used were 5 mmol/L acetic ammonium and methanol, and the solvent composition was increased in a linear gradient from 50% organic modifier to 85% at 7 min. The flow rate was 0.25 mL/min, the column oven temperature was 40 °C, and the injection volume was kept at 2 µL (administration study) or 0.1 µL (permeability study). MS was performed using the Agilent 6470 Triple Quadrupole LC/MS system (Agilent Technology, Ltd.). The ion source was the Agilent Jet Stream (AJS) (Positive/Negative mode), and the drying gas temperature and flow rate were 250 °C and 10 L/min, respectively, while the sheath gas temperature and flow rate were 400 °C and 12 L/min, respectively. The fragmentor voltage was 140 V, and the nozzle voltage was 1000 V. MRM transition was performed at $m/z = 403 > 139$ (30 eV), 297 (15 eV).

To detect metabolites by incubation using S9 supplemented with NADP, dried samples were re-dissolved in methanol. The LC system and analytical column were the same as described above. The mobile phases used were 0.1% formic acid and methanol, and the solvent composition was changed in a linear gradient from 10% methanol to 100% methanol in 30 min. The flow rate was 0.2 mL/min. The quantification of CTVD and the search for metabolites of CTVD were performed using an Agilent 6545 quadrupole time-of-flight mass spectrometer (Q-TOF) LC/MS system (Agilent Technologies, Ltd.). The drying gas temperature and fragmentor voltage were 350 °C and 120 V, respectively, and the other conditions were as described above. Screening for metabolites was based on a database of predicted metabolites (Table A2). Then, peaks from control were excepted.

To detect CTVD glucuronide by incubation of S9 with UDPGA, dried samples were redissolved in acetonitrile. MS was performed using the Agilent 6530 Q-TOF LC/MS system (Agilent Technology,

Ltd.). The mobile phases used were 5 mmol/L acetic ammonium and methanol, and the solvent composition was increased in a linear gradient from 10% organic modifier to 100% at 30 min. The flow rate was 0.2 mL/min, the column temperature was 40 °C, and the injection volume was 3 µL. the ion source was the AJS (Positive mode). Other conditions were as described above.

4.6. Statistical Analyses

The mean and SD of each value were calculated. In incubation with S9, the CTVD concentration and metabolite generation rate in humans and swine were analyzed using Student's *t*-test or Welch's test. *p* values of <0.05 were considered to indicate statistical significance. All statistical analyses were performed using R version 3.5.0 (2018-04-23) (R Core Team [2018]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. available from: https://www.R-project.org/).

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Conflicts of Interest: The authors declare no conflict of interest in association with the present study.

Appendix A

Table A1. Information of mass spectrum for each metabolite.

Metabolites	Retention Time (min)	Polarity	Base Peak Ion	Mass Accuracy
				Relative Mass Error
Hydroxylation and methylation	25.10	Positive	$(M + H)^+$	2.9
Desaturation	25.79	Positive	$(M + H)^+$	1.1
Dihydroxylation	22.57	Positive	$(M + H)^+$	3.1

Table A2. The database of predicted metabolites of CTVD.

Name	Chemical Formula	Exact Mass	Name	Chemical Formula	Exact Mass
(O, N, S) Methylation	$C_{24}H_{32}O_6$	416.21989	Ethyl to Carboxylic Acid	$C_{22}H_{26}O_8$	418.16277
1,4-Dihydropyridines to Pyridines	$C_{23}H_{28}O_6$	400.18859	First/Second Alcohols to Aldehyde/Ketone	$C_{23}H_{28}O_6$	400.18859
2-Ethoxyl to Acid	$C_{22}H_{26}O_7$	402.16785	Glucuronidation + Hydrogenation	$C_{29}H_{40}O_{12}$	580.25198
2 × Glucuronide Conjugation	$C_{35}H_{46}O_{18}$	754.26841	Glucuronide Conjugation	$C_{29}H_{38}O_{12}$	578.23633
2 × Hydrogenation	$C_{23}H_{34}O_6$	406.23554	Glutamine Conjugation	$C_{28}H_{38}NO_8$	516.25974
2 × Hydroxylation and Sulfation	$C_{23}H_{30}O_{14}S_2$	594.10770	Gluthation Conjugation	$C_{33}H_{47}N_3O_{12}S$	709.28804
2 × Hydroxylation	$C_{23}H_{30}O_8$	434.19407	Gluthation Conjugation + Demethylation	$C_{32}H_{43}N_3O_{12}S$	693.25674

Table A2. *Cont.*

Name	Chemical Formula	Exact Mass	Name	Chemical Formula	Exact Mass
2 × Hydroxylation + 2 × Glucuronide	$C_{35}H_{46}O_{20}$	786.25824	Gluthation Conjugation + Dihydroxylation	$C_{33}H_{45}N_3O_{14}S$	739.26222
2 × Oxidation + Glucuronidation	$C_{29}H_{38}O_{14}$	610.22616	Gluthation Conjugation + Hydroxylation	$C_{33}H_{45}N_3O_{13}S$	723.26731
2 × Sulfate Conjugation	$C_{23}H_{30}O_{12}S_2$	562.11787	Gluthation Conjugation, Hydroxylation + Oxidation	$C_{33}H_{43}N_3O_{14}S$	737.24657
3 × Hydroxylation	$C_{23}H_{30}O_9$	450.18898	Glycine Conjugation	$C_{25}H_{33}NO_7$	459.22570
3 × Oxidation + Dehydrogenation	$C_{23}H_{28}O_9$	448.17333	Hetero oxide reduction + Hydrogenation	$C_{23}H_{32}O_5$	388.22497
Acetylation	$C_{25}H_{32}O_7$	444.21480	Hydration, Hydrolysis (Internal)	$C_{23}H_{32}O_7$	420.21480
Acetylation + Oxidation	$C_{25}H_{32}O_8$	460.20972	Hydrogenation	$C_{23}H_{32}O_6$	404.21989
Alcohols Dehydration	$C_{23}H_{28}O_5$	384.19367	Hydrolysis + 2× Oxidation	$C_{23}H_{32}O_9$	452.20463
Alkene to Epoxide	$C_{23}H_{30}O_7$	418.19915	Hydroxylation	$C_{23}H_{30}O_7$	418.19915
Alkenes to Dihydrodiol	$C_{23}H_{32}O_8$	436.20972	Hydroxylation + Glucuronide	$C_{29}H_{38}O_{13}$	594.23124
Aromatic Oxidation	$C_{24}H_{32}O_8$	448.20972	Hydroxylation and Dehydration	$C_{23}H_{28}O_6$	400.18859
Aromatic Ring to Arene Oxide	$C_{23}H_{30}O_7$	418.19915	Hydroxylation and Desaturation	$C_{23}H_{28}O_7$	416.18350
Carboxylation + Glucuronidation	$C_{29}H_{36}O_{14}$	608.21051	Hydroxylation and Ketone Formation	$C_{23}H_{28}O_8$	432.17842
Cysteine Conjugation	$C_{26}H_{37}NO_8S$	523.22399	Hydroxylation and Methylation	$C_{24}H_{32}O_7$	432.21480
Cysteine Conjugation and Desaturation	$C_{26}H_{35}NO_8S$	521.20834	Hydroxylation and Sulfation	$C_{23}H_{30}O_{10}S$	498.15597
Cysteine Glycine Conjugation	$C_{28}H_{40}N_2O_9S$	580.24545	Hydroxymethylene Loss	$C_{22}H_{28}O_5$	372.19367
Cysteine Glycine Conjugation and Desaturation	$C_{28}H_{38}N_2O_9S$	578.22980	Isopropyl Dealkylation	$C_{20}H_{24}O_6$	360.15729
Deacetylation	$C_{21}H_{28}O_5$	360.19367	Isopropyl to Acid	$C_{21}H_{24}O_8$	404.14712
Dacetylation + Dehydrogenation	$C_{21}H_{26}O_5$	358.17802	Isopropyl to Alcohol	$C_{20}H_{24}O_7$	376.15220
Debenzylation	$C_{16}H_{24}O_6$	312.15729	Ketone to Alcohol	$C_{23}H_{32}O_6$	404.21989
Debutylation + Hydrogenation	$C_{19}H_{24}O_6$	348.15729	Methyl Ketone to Acid	$C_{21}H_{26}O_7$	390.16785
Decarbonylation	$C_{22}H_{30}O_5$	374.20932	Methylene to Ketone	$C_{23}H_{28}O_7$	416.18350
Decarboxylation	$C_{22}H_{30}O_4$	358.21441	N-Acethylcysteine Conjugation	$C_{28}H_{39}NO_9S$	565.23455
Decarboxylation and Glucuronidation	$C_{28}H_{38}O_{11}$	550.24141	N-Acethylcysteine Conjugation and Desaturation	$C_{28}H_{37}NO_9S$	563.21890
Deethylation	$C_{21}H_{26}O_6$	374.17294	Oxidation + 2× Desaturation	$C_{23}H_{26}O_7$	414.16785
Demethylation	$C_{22}H_{28}O_6$	388.18859	Oxidation + Acethylcysteination	$C_{28}H_{39}NO_{10}S$	581.22947
Demethylation + Dehydrogenation	$C_{22}H_{26}O_6$	386.17294	Oxidation + Deacetylation	$C_{21}H_{28}O_6$	376.18859
Demethylation + Glucuronidation	$C_{28}H_{36}O_{12}$	564.22068	Oxidation + Demethylation + Dehydrogenation	$C_{22}H_{26}O_7$	402.16785

Table A2. *Cont.*

Name	Chemical Formula	Exact Mass	Name	Chemical Formula	Exact Mass
Demethylation + Hydrogenation	$C_{22}H_{30}O_6$	390.20424	Parent	$C_{23}H_{30}O_6$	402.20424
Demethylation + Oxidation + Glucuronidation	$C_{28}H_{36}O_{13}$	580.21559	Phosphorylation	$C_{23}H_{31}O_9P$	482.17057
Demethylation and Hydroxylation	$C_{22}H_{28}O_7$	404.18350	Propyl Ether to Acid	$C_{20}H_{22}O_7$	374.13655
Demethylation and Methylene to Ketone	$C_{22}H_{26}O_7$	402.16785	Propyl Ketone to Acid	$C_{19}H_{22}O_7$	362.13655
Demethylation and two Hydroxylations	$C_{22}H_{28}O_8$	420.17842	Quinone Formation	$C_{23}H_{28}O_8$	432.17842
Demethylation to Carboxylic Acid	$C_{23}H_{28}O_8$	432.17842	Sulfate Conjugation	$C_{23}H_{30}O_9S$	482.16105
Desaturation	$C_{23}H_{28}O_6$	400.18859	Taurine Conjugation	$C_{25}H_{35}NO_8S$	509.20834
Desaturation + Gluthation Conjugation	$C_{33}H_{45}N_3O_{12}S$	707.27239	Tert-Butyl Dealkylation	$C_{19}H_{22}O_6$	346.14164
Epoxidation + Gluthation Conjugation	$C_{33}H_{47}N_3O_{13}S$	725.28296	Tert-Butyl to Acid	$C_{20}H_{22}O_8$	390.13147
Ethyl Ether to Acid	$C_{21}H_{24}O_7$	388.15220	Tert-Butyl to Alcohol	$C_{19}H_{22}O_7$	362.13655
Ethyl Ketone to Acid	$C_{20}H_{24}O_7$	376.15220	Triphosphorylation	$C_{23}H_{33}O_{15}P_3$	642.10323
Ethyl to Alcohol	$C_{21}H_{26}O_7$	390.16785	Two Sequential Desaturations	$C_{23}H_{26}O_6$	398.17294

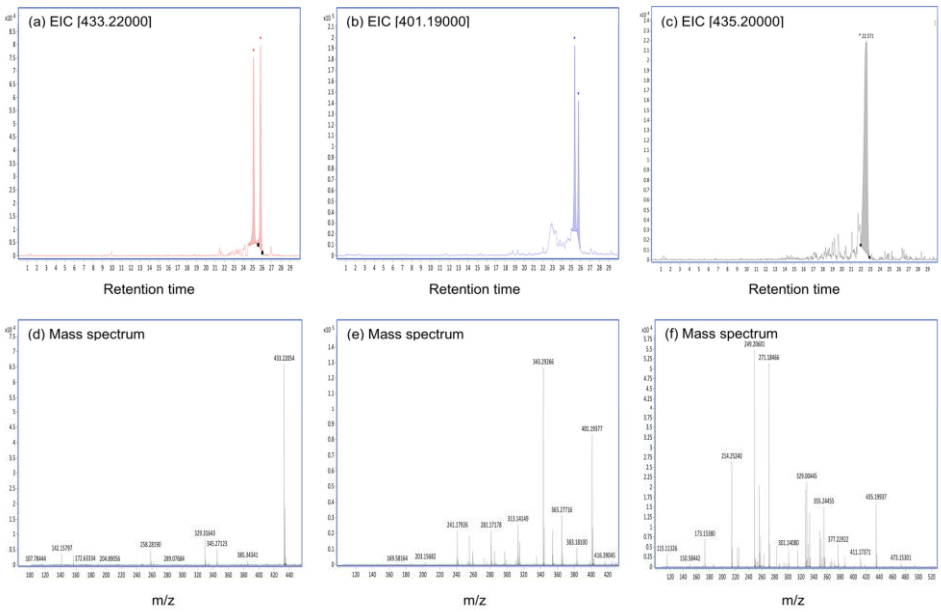

Figure A1. The extracted ion chromatogram (EIC) and mass spectra of the main metabolites of CTVD incubated with S9. The EIC and mass spectra were analyzed by Q-TOF. The compounds presumably produced by incubation with S9 fractions supplemented with NADP were as follows: (**a–c**) represent the EIC of hydroxylation and methylation (EIC 433.22000), desaturation (EIC 401.19000), and dihydroxylation (EIC 435.20000), respectively, at 240 min after incubation with swine hepatic S9. (**d–f**) represent their mass spectra.

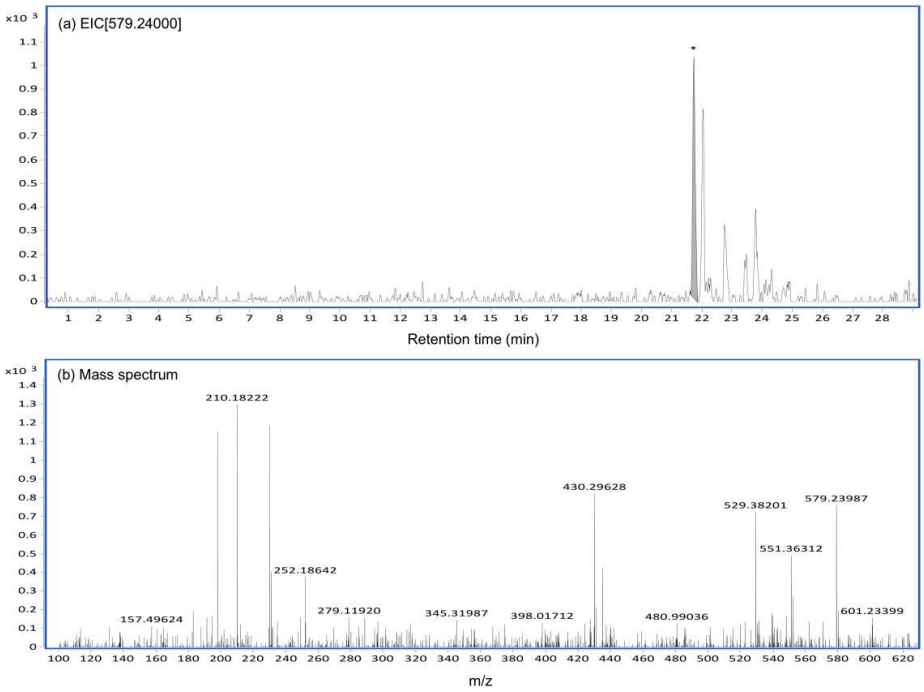

Figure A2. The EIC and mass spectrum of CTVD glucuronide generated by incubation with S9 including UDPGA. The EIC and mass spectrum of CTVD glucuronide produced by incubation with swine hepatic S9 supplemented with UDPGA were analyzed by Q-TOF. The EIC (**a**) and mass spectrum (**b**) of CTVD glucuronide as observed at 240 min after incubation with swine hepatic S9.

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出　典

本論文は以下に公表した。

・Uchiyama, Y.; Takino, M.; Noguchi, M.; Shiratori, N.; Kobayashi, N.; Sugita-Konishi, Y. The In Vivo and In Vitro Toxicokinetics of Citreoviridin Extracted from *Penicillium citreonigrum*. *Toxins* **2019**, *11*, 360.
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