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In vitro phase I metabolism of vinclozolin by human liver microsomes

Marycarmen Cruz-Hurtado,[1] Ma. de Lourdes López-González,[1] Victor Mondragón,[2] and Adolfo Sierra-Santoyo[1]*

[1]Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav), Av. IPN No. 2508, Col. San Pedro Zacatenco, Ciudad de México, CP 07360, México. [2]Centro de Excelencia de Agilent Technologies México, Av. Insurgentes Sur No. 1602, Oficina 801. Col. Crédito Constructor, Ciudad de México, C.P. 03940, México.

*Corresponding author: Adolfo Sierra-Santoyo, Centro de Investigación y de Estudios Avanzados del IPN (Cinvestav-IPN), Departamento de Toxicología, Av. IPN No. 2508, Col. San Pedro Zacatenco, Ciudad de México, CP 07360, México. Telephone: (52) 55 57473800, ext. 5425. Fax: (52) 55 57473395. E-mail: asierra@cinvestav.mx

Abstract

1. Vinclozolin (Vin) is a fungicide used in agricultural settings and is classified as an endocrine disruptor. Vin is non-enzymatically hydrolyzed to 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) metabolites. There is no information about Vin biotransformation in humans, therefore, the aim of this study was to characterize its *in vitro* metabolism using human liver microsomes.
2. Vin was metabolized to the [3-(3,5-dichlorophenyl)-5-methyl-5-(1,2-dihydroxyethyl)-1,3-oxazolidine-2,4-dione] (M4) and N-(2,3,4-trihydroxy-2-methyl-1-oxo)-3,5-dichlorophenyl-1-carbamic acid (M7) metabolites, which are unstable and gradually converted to 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide (DTMBA, formerly denoted as M5). M4 and DTMBA metabolites co-eluted in the same HPLC peak; this co-elute peak exhibited a Michaelis-Menten kinetic, whereas M7 showed a substrate inhibition kinetics. The $K_{M\ app}$ for co-eluted M4/DTMBA and M7 was 24.2±5.6 and 116.0±52.6 μM, the $V_{Max\ app}$ was 0.280±0.015 and 0.180±0.060 nmoles/min/mg protein, and the $CL_{int\ app}$ was 11.5 and 1.5 mL/min/g protein, respectively. The K_i for M7 was 133.2±63.9 μM. Cytochrome P450 (CYP) chemical inhibitors furafylline (CYP1A2), ketoconazole (CYP3A4), pilocarpine (CYP2A6), and sulfaphenazole (CYP2C9) inhibited M4/DTMBA and M7 formation, suggesting that Vin is metabolized in humans by CYP.
3. DTMBA is a stable metabolite and specific of Vin, therefore it could be used as a biomarker of Vin exposure in humans to perform epidemiological studies.

Key words: Vinclozolin; cytochrome P450; antiandrogen; biomarkers; xenobiotic metabolism.

Introduction

Pesticides are a heterogeneous group of chemicals to control and repel pests in different fields. Vinclozolin (Vin) is a nonsystemic dicarboximide fungicide used to prevent rotting of several fruits, vegetable, ornamentals plants, and turf glass (Szeto *et al.* 1989, US EPA 2000). Human exposure to Vin can occur via ingestion of contaminated produce food and drink or from occupational use.

In rats, Vin is efficiently metabolized by first undergoing a non-enzymatic hydrolysis to generate 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) metabolites (Szeto *et al*. 1989; Sierra-Santoyo *et al*. 2004). Regarding phase I metabolism, Vin is metabolized to at least seven metabolites by reactions catalyzed by the cytochrome P450 (CYP) 2A, 2B, and 3A subfamilies (Sierra-Santoyo *et al*. 2012). Vin is metabolized to [3-(3,5-dichlorophenyl)-5-methyl-5-(1,2-dihydroxyethyl)-1,3-oxazolidine-2,4-dione] (M4) and N-(2,3,4-trihydroxy-2-methyl-1-oxo)-3,5-dichlorophenyl-1-carbamic acid (M7) metabolites; these products are unstable in physiological conditions and are finally converted to 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide (DTMBA, formerly denoted as M5 metabolite) (Fig. 1). DTMBA represents the main metabolic product of Vin present in organs and tissues, except in the adipose tissue, and may also be formed from M2 metabolite (Sierra-Santoyo *et al*. 2008). Bursztyka *et al* (2008), proposed that M1 is hydroxylated in its ethylene group to form a metabolite designated as M6 and converted to DTMBA via ring closure to form M4. The 3,5-dichloroaniline (M3) metabolite formation has been reported as a product not NADPH-dependent from M2, however, it may not represent an important metabolite in mammals (Sierra-Santoyo *et al*. 2012). With respect to phase II metabolism, glucuronide- and sulfate-DTMBA and M4 conjugates have been reported as the main Vin metabolites excreted in urine (Bursztyka *et al*. 2008, Rathahao-Paris *et al*. 2014, Cruz-Hurtado *et al*. 2018).

Although Vin exhibits low acute toxicity to wildlife (US EPA, 2000), the administration of Vin to pregnant rats at specific gestational stages has produced a significant degree of morphological changes, feminization, and demasculinization of male offspring (Gray *et al.* 1994, Monosson *et al.* 1999, Wolf *et al.* 2000), as well as behavioral and reproductive alterations in adult males in other species (Moorman *et al.* 2000, Veeramachaneni *et al.* 2006, Nakamura *et al.* 2014, Huang *et al.* 2015;). These anti-androgenic effects have been associated with Vin and its metabolites M1 and M2 (Kelce *et al*. 1994, 1997). The androgen receptor (AR) is competitively inhibited by Vin, M1, and M2 (Kelce *et al*. 1994, Wong *et al*. 1995) and this inhibition interferes with androgen-dependent gene expression (Gray *et al*. 1994, Monosson *et al*. 1999, Wolf *et al*. 2000, Molina-Molina *et al*. 2006). However, *in vivo* antiandrogenic effects cannot be completely explained by the tissue dosimetry of Vin and/or its metabolites (Sierra-Santoyo *et al*. 2008). In this regard, a recent *in silico* study has shown that isomers of DTMBA can bind to AR of humans, rats, and zebrafish and compete with endogenous hormones, generating antiandrogenic effects (Galli *et al*. 2014).

Regarding Vin toxicity in humans, there is only one report in which M3 was used as a biomarker of Vin exposure, but no association between anti-androgenic or reproductive effects and urinary M3 levels was reported in an occupational setting of Vin exposure (Zober *et al*. 1995). The absence of an association in this study may be due to M3 is not a specific metabolite of V since it is also produced from other dicarboximides, such as procymidone, iprodione, and chlozolinate (Lindh et al., 2007; Wittke et al., 2001). In a pilot study conducted in a rural province of China (Zhejiang), in which 20 non-persistent pesticides were analyzed by gas chromatography-mass spectrometry, Vin and acetochlor cord blood levels were significantly associated with reduced birth weight (Wickerham *et al*. 2012). Despite the limited information in humans and assuming a similar *in vivo* dose-response relationship and a temporal association

supported by its mechanism of action, Vin effects observed on the reproductive tract of male animals are highly plausible in humans (Kavlock and Cummings 2005). This proposal is supported by the fact that despite Vin instability in physiological conditions, it has been identified in blood of umbilical cords and adult people, and placenta samples (Molina-Molina *et al.* 2006, Wickerham *et al.* 2012).

Presently, there is no available information about Vin metabolism in humans. Thus, extrapolation of the pathway described in rats to humans seems remote because the CYP isoforms involved in its biotransformation have differences in catalytic activities in each specie. The lack of information about Vin pharmacokinetics in humans indicates the need for research on the fate of Vin to be biotransformed, accumulated, and excreted in humans and to determine effects on the male reproductive tract associated with Vin exposure. Thus, the aim of this study was to know the phase I biotransformation of Vin by human liver microsomes (HLM).

Materials and Methods

Chemicals

Vin (99.96% purity, lot 4355400) was purchased from Chem Service Inc. (West Chester, PA, USA). HLM (male pool of 10, H100 Lot. 1210270) were purchased from Xenotech (Lenexa, KS, USA). M1 and M2 metabolites were isolated as described by Szeto et al. (1989). DTMBA was isolated from urine Vin-treated rats as described by Cruz-Hurtado *et al*. (2018). Methanol, acetonitrile, and water were HPLC-grade and were purchased from Burdick and Jackson, Inc (Muskegon, MI, USA). Furafylline, quinidine, ketoconazole, sulfaphenazole, M3, pilocarpine, and nicotinamide adenine dinucleotide (NAPDH) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). The policlonal anti-human CYP3A4 and 2C9 antibodies were purchased from Abcam PLC (Cambridge, U.K.). The polyclonal anti-human CYP1A2 and 2D6

antibodies were obtained from Chemicon International Inc. (Temecula, CA, USA). All other chemicals were of the highest purity available.

Enzyme assays by human liver microsomes.

The *in vitro* metabolism assays of Vin by HLM were carried out as previously described by Sierra-Santoyo *et al*. (2012), with some modifications. Briefly, Vin (50 μM) and HLM were incubated in a final volume of 1 mL of 100 mM KH_2PO_4 and 5 mM $MgCl_2$ buffer at pH 7.4 in a shaking water bath at 37°C. The linear conditions for Vin metabolism by HLM to determine the enzyme kinetic parameters were experimentally established at 1 mg/mL of microsomal protein, 1 mM NADPH, and an incubation time of 30 min (data not shown). Reactions were started by NADPH addition. After the incubation time, the reactions were finalized by the addition of 5 mL cold acetonitrile to 100 μL of incubation media, then vortexed and centrifuged (1,650xg) at 4°C for 10 min. The supernatants were dried using sodium sulfate and evaporated under a stream of N_2 at 45°C. Samples were stored at 4°C and reconstituted in 100 μL of acetonitrile just before the chromatographic analysis. Blanks at zero time, of CO-bubbled HLM, and without NADPH were carried out using the same enzyme assay conditions and processed for the analysis of metabolites. All samples were prepared in triplicate and repeated three times.

Inhibition enzyme assays

To determine which CYPs are involved in Vin metabolism five CYP chemical inhibitors and four anti-human CYP antibodies were tested. Inhibition assays were tested for CYP1A2, 2A6, 2C9, 2D6, and 3A4, with exception of CYP2E1; these isoforms are the main human liver CYPs involved in xenobiotic metabolism. Chemical inhibitors were preincubated in similar conditions

to those established for the enzyme assay using 50 μM Vin as substrate. Inhibitors were added to the medium enzyme assay in a maximum volume of 20 μL of methanol at the following concentrations: 0.5-3 μM furafylline (CYP1A2), 0.5-4 μM pilocarpine (CYP2A6), 0.6-1.5 μM sulfaphenazole (CYP2C9), 0.06-0.2 μM quinidine (CYP2D6), and 0.002-0.2 μM ketoconazole (CYP3A4) (Kinonen *et al*. 1995, Khojastech *et al*. 2011). Methanol concentration in the incubation media did not affect the CYP enzyme activities.

Inhibition assays were complemented using anti-human CYP1A2, 2C9, 2D6 and 3A4 antibodies in a final volume of 0.2 mL. For each CYP evaluated, antiserum was diluted 1:25. The control assay without antibody and assays with antibodies. All assays were done in duplicate and repeated twice.

Chromatographic analytical method

Vin metabolism products were quantified by HPLC/DAD using chromatographic conditions previously reported by Sierra-Santoyo *et al*. (2004) with some modifications. Briefly, a Zorbax Eclipse XDB-C18 column (5 μm particle size; 4.6 mm ID × 250 mm, 80 Å) (Agilent Technologies, Little Falls, DE, USA). System solvents consisted of methanol:acetonitrile 70:30 (A) and 0.02 M monobasic sodium phosphate pH 3.3 (B). The initial solvent conditions were set at 40% A:60% B at a flow of 0.6 mL/min. After sample injection there was a 20 min linear gradient change to 20% A: 80% B. Finally, there was a 5 min linear gradient change of 40% A:60% B to reestablish initial conditions. The detector wavelength was set at 212 nm and the reference wavelength was 550 nm. These changes did not significantly modify the recovery and precision of Vin metabolites as described previously (Sierra-Santoyo *et al*. 2004). Vin metabolites were characterized by UPLC/MSD using an Agilent Model 1290 Infinity II coupled

to a MS Q-TOF model 6545 (Agilent Technologies, Santa Clara, CA). The chromatographical and operation conditions and the mass spectrometer was operated in negative electrospray ionization (ESI) mode as described by Cruz-Hurtado *et al.* (2018).

Calibration graphics of Vin, M1, M2, and DTMBA were prepared in acetonitrile. Concentration of metabolites M4/DTMBA and M7 were estimated using the DTMBA calibration graphic.

Data analysis

Data of M4/DTMBA were fitted to a one-enzyme Michaelis-Menten equation $V=V_{max}*S/K_m+S$. M7 metabolite plot of V versus S showed a substrate inhibition kinetic, data were fitted following the equation $V=V_{max}*S/K_m+S\ (1+S/K_i)$. Eadie-Hofstee transformations plots were made to identify the behavior of the enzyme kinetics models (Venkatakrishnan *et al*. 2001, Wu 2011). Apparent kinetic parameters, $K_{m\ app}$, $V_{max\ app}$, and K_i for the substrate inhibition kinetic were calculated by nonlinear regression using GraphPad Prism ver. 4.01 (GraphPad Software Inc., San Diego, CA, USA).

Results

Because there is no information about nonenzymatic hydrolysis of Vin in human biological samples, the stability of the fungicide in the presence or absence of HLM was determined. In both conditions, Vin was gradually non-enzymatically hydrolyzed, its $t_{1/2}$ in the absence of microsomal protein was 63 min, while in the presence of microsomal protein, its $t_{1/2}$ decreased to 38.1 min. On the contrary, the concentration of the metabolites M1 and M2 increased over time, and M2 levels remained constant after 30 min in the presence of HLM (Fig. 2).

Incubating Vin in the presence of HLM resulted in the formation of two new peaks, in addition to M1 and M2, which eluted at 16.8 and 17.9 min, respectively. The new peaks eluted at 10.3 and 12.4 min, which were designated as M4/DTMBA and M7, respectively (Fig. 3B). Only Vin and low levels of M1 and M2 were detected in the incubation media that lacked NADPH, or were either heat- or CO-treated or at zero-time. In a similar way, as previously reported using rat liver microsomes (Sierra-Santoyo *et al*. 2012), the peak at 10.3 min corresponded to the co-elution of two metabolites. In fresh samples immediately processed, this peak showed an UV spectrum similar to Vin in the ascending section corresponding to M4 (Fig. 3B, inset a) and other in the descending section similar to DTMBA (Fig. 3B, inset b). M4 and DTMBA metabolites were identified by negative ESI mass spectrometry analysis (Fig 4). The M4 metabolite consisted of molecular ions at *m*/*z* 317.9960 $[M\text{-}H]^-$, *m*/*z* 274.0042 $[M\text{-}CO_2]^-$, *m*/*z* 246.0111 $[M\text{-}C_4H_8O_2\text{+}H_2O]^-$, and *m*/*z* 159.9725 $[M\text{-}C_6H_8\ O_5]^-$. The negative ESI data of DTMBA showed molecular ions at *m*/*z* 292.0156 $[M]^-$, *m*/*z* 231.9934 $[M\text{-}C_2H_5O_2]^-$, and *m*/*z* 159.9725 $[M\text{-}C_5H_{10}O_4]^-$. Based on these spectroscopic results, these metabolites were identified as M4 and DTMBA, which have a MW of 318 and 293, respectively. The metabolite M7 is an unstable intermediate that we were not able to identify.

After the identification of Vin metabolic products, the enzymatic kinetics of M4/DTMBA and M7 metabolites were determined using the conditions for their linear formation by incubation for 30 min at 37°C. Results of the enzyme kinetic of M4/DTMBA metabolites showed a simple monophasic and hyperbolic kinetic (r=0.976), which was verified by the Eadie-Hofstee plot and fit to the Michaelis-Menten plot (Fig. 5). Results of the enzyme kinetic analysis of M7 showed a substrate inhibition kinetic, which was verified by the Eadie-Hofstee plot. The calculated $K_{M\ app}$ corresponding to the co-elution peak M4/DTMBA (24.2 ± 5.6 μM) was lower than that calculated

for M7 (116.0 ± 52.6 μM). The $V_{Max\ app}$ values for M4/DTMBA and M7 were 0.280 ± 0.015 and 0.184 ± 0.060 nmoles of product/min/mg protein, respectively. The intrinsic clearance ($CL_{int\ app}=V_{Max\ app}/K_{M\ app}$) values for M4/DTMBA and M7 were 11.5 and 1.5 mL/min/g protein, respectively, and the inhibitory constant (K_i) calculated for M7 in the inhibitory substrate kinetic was 133.2 ± 63.9 μM.

To determine which were the main CYPs involved in Vin metabolism, five chemical inhibitors for the main human liver CYPs were tested. Furafylline and ketoconazole were the main inhibitors of the formation of M4/DTMBA and M7 metabolites. The half inhibitory concentrations (IC_{50}) of ketoconazole for M4/DTMBA and M7 formation were 0.280 and 0.091 μM, respectively; the IC_{50} of furafylline for M4/DTMBA and M7 was 2.2 and 1.3 μM, respectively. Pilocarpine and sulfaphenazole strongly inhibited the M7 formation, the IC_{50} was 0.227 and 1.247 μM, respectively (Fig. 6). The addition of anti-human CYP1A2, 2C9, and 3A4 antibodies to the incubation media at the dilution assayed completly inhibited the production of M4/DTMBA and M7 metabolites. Vin metabolism was not affected by the addition of quinidine or anti-human CYP2D6 antibody to the incubation medium.

Preliminary studies on M1 and M2 metabolism indicate that M2 was metabolized to M5, and trace levels of M3 were detected from 100 μM of substrate. M1 was not metabolized under the enzyme assay conditions used (results not shown).

Discussion

The main finding of this study was that phase I Vin metabolism in humans is CYP-dependent and its metabolic pathway is similar to that described for rats (Fig. 1). Vin was non-enzymatically hydrolyzed to M1 and M2 metabolites in the presence and absence of HLM (Fig. 2). However,

the Vin hydrolysis in the presence of microsomal protein enhanced. These results confirm that Vin is very susceptible to hydrolysis in basic media and is favored by the presence of proteins, lipids and ions, as has been previously reported when Vin was incubated in phosphate buffer pH 7.4 and rat serum (Szeto *et al*. 1989, Sierra-Santoyo *et al*. 2004). Likewise, Vin is metabolized to M4 and M7 metabolites, which are unstable and spontaneously converted to DTMBA by some CYPs. It is possible that Vin undergoes a CYP-dependent epoxidation reaction on the vinyl group to generate the epoxide-M4, followed by an epoxyde hydrolase reaction or by spontaneous hydrolysis to form the M4 metabolite, and the opening of the 2,4-oxazolidinedione ring by the addition of water resulting in the formation of M7, which undergoes decarboxylation to finally form DTMBA. Another pathway of biotransformation is through M7, bypassing M4 and undergoing a rearrangement to finally form DTMBA.

DTMBA was the most stable metabolite of Vin; in incubations longer than 30 min, the disappearance of M4 and M7 was remarkable, which resulted in DTMBA as the final product. These results are in agreement with those described in *in vitro* and *in vivo* studies in the rat, in which DTMBA was the main Vin metabolite of phase I (Bursztyka *et al*. 2008, Sierra-Santoyo *et al*. 2008, 2012). Likewise, DTMBA could also be generated from M2 by an enzymatic epoxidation of the ethylene group followed by the hydrolysis of the epoxide (Sierra-Santoyo *et al*. 2008). To date, we do not have evidence about the formation of the intermediary epoxide either from Vin or M2 metabolite. In this study, the M3 metabolite was not detected as a product of Vin biotransformation by HLM. A possible explanation could be that it is generated at very low concentrations and could only be originated from the M2 biotransformation. This possibility is supported by our preliminary studies detecting M3 at trace levels from 100 μM of M2 as substrate (results not shown). Currently, there is no information about M1 and M2 metabolism in

humans as yet; therefore, further studies are needed to determine the metabolic fate of M1 and M2 in humans, and if M3 is formed from M2, as it was reported using rat liver microsomes (RLM) (Sierra-Santoyo *et al*. 2012). Although not fully addressed, preliminary studies on enzymatic hydrolysis of Vin, M1, and M2 using hepatic or serum esterases obtained from rat or human have shown lack of enzymatic activity (results not shown). These results also proved that M3 is a minor metabolic product and is not a specific metabolite of Vin, which indicates that it does not meet the necessary criteria to be used as biomarker of Vin exposure.

In the current study, we demonstrated that Vin is metabolized in humans by liver microsomal CYP-dependent monooxygenases. The results of M4/DTMBA and M7 formation using chemical inhibitors and anti-human CYP antibodies indicated a differential effect on each metabolite. Furafylline, a human CYP1A2 inhibitor (Ono *et al*. 1996), inhibited both M4/DTMBA and M7 formation, showing IC_{50} values of 2.2 and 1.348 μM, respectively. These values are within the range of the *Ki* values (0.6-4.4 μM) determined by phenacetin *O*-deethylase activity (Bourrié *et al*. 1996, McKillop *et al*. 1998). Ketoconazole has been recognized as a powerful inhibitor of CYP3A subfamily and other CYPs in several species including the human (Ono *et al*. 1996, Khojastech *et al*. 2011). In this study, ketoconazole inhibited M4/DTMBA and M7 formation, with an IC_{50} of 0.287 and 0.091 μM, respectively. These values are consistent with those reported for azamulin, a potent and selective inhibitor of CYP3A (Stresser *et al*. 2004). In contrast, sulfaphenazole and pilocarpine inhibited M7 formation; sulfaphenazole is a potent and very selective inhibitor of CYP2C subfamily, although CYP2C9 is the most susceptible isoform to be affected (Ono *et al*. 1996, Khojastech *et al*. 2011). Our data showed that sulfaphenazol had an IC_{50} of 1.247 μM; this value is very close to the *Ki* value reported for CYP2C9 determined by the tolbutamide 4-hydroxylase activity (Bourrié *et al*. 1996, Komatsu *et*

al. 2000). Pilocarpine has been shown to inhibit CYP2A, 2B, and 3A activities in human and mouse (Kinonen *et al*. 1995). The IC_{50} of pilocarpine calculated in this study (0.227 μM) is much lower than the IC_{50} calculated for Vin as substrate using RLM (22.6 μM) (Sierra-Santoyo *et al*. 2012) and for 7-hydroxylation of coumarin using HLM (4 μM) (Bourrié *et al*. 1996). Together, these results suggest that CYP1A2, 2A6, 2C9, and 3A4 could be the main enzymes involved in Vin biotransformation in humans. This proposal is supported by the results observed when anti-human CYP antibodies were added to the enzymatic assay medium. These results represent the first approach to determine CYPs involved in Vin metabolism in humans. Further studies are needed to determine a more reliable individual contribution using recombinant heterologously-expressed human CYPs.

This is the first report describing kinetic parameters, such as $K_{M\ app}$, $V_{Max\ app}$, and $CL_{int\ app}$ of Vin biotransformation by HLM. Rane *et al* (1977) proposed that the *in vitro* CL_{int} may be used to link the fundamental enzymatic kinetics (K_M and V_{Max}) and *in vivo* pharmacokinetic variables, in addition it may help to predict the rate of hepatic clearance *in vivo* and to define the contribution of individual metabolic pathways when a substrate is metabolized to more than two products (Obach 1999, Sjögren *et al*. 2009). In this study, the enzymatic kinetic parameters were individually obtained despite the instability of the intermediary metabolites in a similar manner as they were calculated for RLM (Sierra-Santoyo *et al*. 2012). The $CL_{int\ app}$ for M4/DTMBA was 7.6-fold higher than that of M7, which indicates a preferential formation of M4/DTMBA from Vin by HLM, similar to that of RLM (Sierra-Santoyo *et al*. 2012). This result suggests that Vin can be easily metabolized to M4 to be finally converted to DTMBA. M7 metabolite formation showed substrate inhibition kinetics; this behavior describes a process in which metabolic rate decreases at high substrate levels and is verified by a low formation of product reflected by a low

value of $CL_{int\ app}$. These results also suggest that in humans, DTMBA could represent the main metabolite in people exposed to Vin because it is the most stable metabolite.

The results of this *in vitro* study using HLM indicate that M3 is not formed from Vin; therefore, M3 is not a specific metabolic product of Vin. In several studies, M3 has been detected in human urine samples (Wittke *et al*. 2001, Weiss and Angerer 2002, Turci *et al*. 2006, Lindh *et al*. 2007, Kutting *et al*. 2009). The presence of M3 in these studies could be due to the acidic or alkaline conditions used for its detection (Szeto *et al*. 1989, Sierra-Santoyo *et al*. 2004). In the only report where M3 was used as a biomarker, there was no association between antiandrogenic or reproductive effects and urinary M3 levels in an occupational setting of Vin exposure (Zober *et al*. 1995). In contrast, in a pilot study conducted in Zhejiang, a rural province in China, Vin and acetochlor cord blood levels were significantly associated with reduced birth weight (Wickerham *et al*. 2012). Molina-Molina *et al* (2006) reported a high frequency of Vin in blood samples obtained from young people living in southern Spain. Based on our results and assuming similar pharmacokinetics of Vin in humans as it was described in rats (Sierra-Santoyo *et al*. 2008), very high levels of other metabolites, mainly DTMBA, should be detected in those human samples. Thus, DTMBA could represent an excellent alternative to be used as a biomarker of Vin exposure because is easly detected after enzymatic hydrolysis of its conjugated metabolites (Bursztyka *et al*. 2008, Rathahao-Paris *et al*. 2014, Cruz-Hurtado *et al*. 2018).

In summary, the current study is the first report describing the *in vitro* Vin metabolism by HLM. Vin was metabolized *in vitro* to M4 and M7 products, which are chemically unstable and by a molecular rearrangement they are transformed to DTMBA. Vin metabolism is CYP-dependent and CYP1A2, 3A4, 2A6, and 2C9, could be the main isoforms involved in its biotransformation. More studies about M1 and M2 metabolism are needed to complete the metabolic pathway of Vin. DTMBA is a stable metabolite and specific of Vin, therefore it may be

used as a biomarker of exposure to Vin in pharmacokinetic and toxicological studies. There is no available toxicological information about M4, DTMBA, or M7 metabolites; then, the toxicological characterization of these metabolites is needed for a better understanding of male reproductive effects in humans associated with V exposure.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Footnotes

Figure 1. Proposed metabolic pathway of Vin by human liver microsomes.

Figure 2. Non-enzymatic hydrolysis of Vin in enzyme assay in the presence or absence of human liver microsomes. (A) depletion profile of Vin; (B) generation of the M1 metabolite; (C) generation of the M2 metabolite. Incubations were carried out using 50 μM Vin in 200 mM KH_2PO_4 and 5 mM $MgCl_2$, pH 7.4 at 37 °C (n=3).

Figure 3. Typical HPLC chromatograms of: (A) standard solutions of Vin (32.5 μM), M1 (16.5 μM), M2 (19 μM), and M3 (31 μM) in acetonitrile; (B) extract of the *in vitro* enzyme assay of 50 μM Vin by human liver microsomes. Inset includes the UV absorption spectra of metabolites M4 (a), DTMBA (b), and M7 (c). Metabolites were extracted in acetonitrile from the enzyme assay incubation medium. Extracts were dried under a stream of N_2, and the residue was reconstituted in 100 μL of acetonitrile. See Materials and Methods section for experimental details.

Figure 4. HPLC/mass spectrometry chromatogram of metabolites generated by the biotransformation of Vin (50 μM) by human liver microsomes (A), negative ionization mass spectra of metabolites DTMBA (B), and M4 (C). System solvents consisted of water:acetonitrile:1% formic acid (70:30:0.1).

Figure 5. Substrate saturation plots of products generated by *in vitro* Vin metabolism by human liver microsomes. (A) M4/DTMBA metabolites, and (B) M7 metabolite. Inserts depict Eadie-Hofstee plots. Vin was incubated in 200 mM KH_2PO_4, 5 mM $MgCl_2$, human liver microsomes (1 mg/mL), and 1 mM NADPH, pH 7.4 at 37 °C during 30 min. Data represent the mean ± standard deviation (n=3).

Figure 6. Effect of chemical inhibitors furafylline (A), pilocarpine (B), sulfaphenazole (C), or ketoconazole (D) on M4/DTMBA and M7 formation from Vin by human liver microsomes. Vin (50 μM) was incubated in 200 mM KH_2PO_4, 5 mM $MgCl_2$, human liver microsomes (1 mg/mL), and 1 mM NADPH, pH 7.4 at 37 °C (n=3).