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Effect of vanillin and ethyl vanillin on cytochrome P450 activity *in vitro* and *in vivo*

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ABSTRACT

Food safety is of extreme importance to human health. Vanillin and ethyl vanillin are the widely used food additives and spices in foods, beverages, cosmetics and drugs. The objective of the present work was to evaluate the impact of vanillin and ethyl vanillin on the activities of CYP2C9, CYP2E1, CYP3A4, CYP2B6 and CYP1A2 in human liver microsomes (HLM) *in vitro*, and impact on the activities of CYP1A2, CYP2C, CYP3A and CYP2E1 in rat liver microsomes (RLM) *in vivo*. The *in vitro* results demonstrated that vanillin and ethyl vanillin had no significant effect on the activity of five human CYP450 enzymes with concentration ranged from 8 to 128 μM. However, after rats were orally administered vanillin or ethyl vanillin once a day for seven consecutive days, CYP2E1 activity was increased and CYP1A2 activity was decreased in RLM. The *in vivo* results revealed that drug interaction between vanillin/ethyl vanillin and the CYP2E1/CYP1A2-metabolizing drugs might be possible, and also suggested that the application of the above additives in foods and drugs should not be unlimited so as to avoid the adverse interaction.

1. Introduction

Cytochrome P450 enzymes (CYPs) comprise a super-gene family and play key roles in the metabolism of drugs, xenobiotics and endogenous compounds in animals and human. Clinically, drug interactions associated with induction or inhibition of CYPs have been shown to be among the important factors in causing side effects in human (Guengerich, 2006). Nowadays, human liver microsomes and recombinant P450 isoforms (CYP2C9, CYP2E1, CYP3A4, CYP2B6, and CYP1A2) are the preferred *in vitro* test system for predicting drug–drug interactions (DDI) (Bjornsson et al., 2003). Because some of CYPs isoforms (such as CYP1A2, CYP2C, CYP3A and CYP2E1) in rat are similar to that of human, rats are also always used to predict human DDI associated with induction or inhibition of CYPs activities *in vivo* (Bogaards et al., 2000).

Vanillin and ethyl vanillin (Fig. 1a and b) are popularly used as the food additives nowadays. Previous studies showed that vanillin is useful as anti-sickle cell anemia (Zhang et al., 2004), anti-mutagen (Sasaki et al., 1990; Ho et al., 2009) and anti-bacteria agent (Rupasinghe et al., 2005) at high concentration of un-oxidized form to be medically effective, as well as antioxidant (Tai et al., 2011). Recently, Ho et al. (2011) reported that vanillin administration at high concentration (150 and 300 mg/kg) had no obvious effect on the expression of most xenobiotic metabolism, cell progression, tumor suppressor, DNA damage and inflammation genes in rat brain, and postulated that vanillin could provide blood and brain protective properties. So far, little is known about the effect of vanillin and ethyl vanillin on the activities of CYPs *in vitro* and *in vivo*. The drug interactions associated with induction or inhibition of CYP enzymes have been shown to be among the important factors in causing side effects in clinics (Guengerich, 1997). The objective of the present work was to evaluate the impact of vanillin and ethyl vanillin on the activities of CYP2C9, CYP2E1, CYP3A4, CYP2B6 and CYP1A2 in HLM *in vitro*, and the impact on the activities of CYP1A2, CYP2C, CYP3A and CYP2E1 in RLM *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was purchased from Roche Co. (Basel, Switzerland). Vanillin and ethyl vanillin were purchased from Acros Organics (New Jersey, USA). Resorufin sodium salt, 4-nitrocatechol and *p*-nitrophenol were purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA). Testosterone and tolbutamide were purchased from Dr. Ehrenstorfer (Ausberg, Germany). Chlorzoxazone was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ethoxyresorufin, nirvanol, 4′-hydroxytolbutamide, (S)-mephenytoin, 6-hydroxychlorzoxazone were purchased from TRC (Toronto, Canada). Phenacetin was purchased from Alfa Aesar (MA, USA). Acetaminophen was purchased from TCI (Tokyo, Japan). 6β-Hydroxytestosterone was purchased from Caymen (Michigan, USA).

Pooled human liver microsomes were purchased from BD Gentest (Woburn, MA, USA). Methanol and acetonitrile (HPLC grade) were purchased from Dikma Company (Beijing, China). All other chemicals and reagents were of analytical grade and were obtained commercially.

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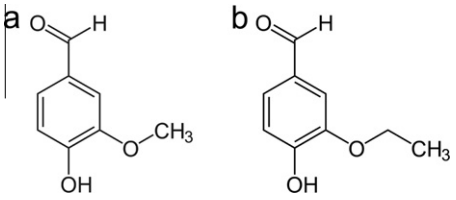

Fig. 1. Structures of vanillin (a) and ethyl vanillin (b).

2.2. In vitro experiments

2.2.1. Incubation in human liver microsomes

All incubations were performed at 37 °C in a system containing human liver microsomal protein (0.5 mg/mL for testosterone 6β-hydroxylation, (S)-mephenytoin *N*-demethylation and chlorzoxazone 6-hydroxylation, 0.75 mg/mL for phenacetin *O*-deethylation, 1 mg/mL for tolbutamide 4′-hydroxylation) and a NADPH regenerating system (NADPH 1 mM, $MgCl_2$ 5 mM) in the final volume of 200 μL (0.1 M potassium phosphate buffer, pH 7.4). After preincubation at 37 °C for 3 min, the incubation reaction was started by the addition of 1 mM NADPH in phosphate buffer. The duration of incubation was 60 min for testosterone and chlorzoxazone, 45 min for phenacetin and tolbutamide, and 30 min for (S)-mephenytoin. The reaction was stopped by cooling on ice and addition of 400 μL methanol (testosterone 6β-hydroxylation, phenacetin *O*-deethylation, tolbutamide 4′-hydroxylation), 800 μL ethyl acetate (chlorzoxazone 6-hydroxylation), 500 μL methanol ((S)-mephenytoin *N*-demethylation). Protein was precipitated by vortexing for 3 min and centrifugation at 12, 000*g* for 10 min, then the supernatant was evaporated (BioTron Ecospin 3180C LABCONCO, Korea) at 40 °C. The residue was stored at −20 °C until HPLC analysis.

The probe substrate concentrations were chosen either at or near the apparent *Km* values of each CYP enzyme (Guengerich, 2006; Bjornsson et al., 2003; Guo and Wang, 2007; Ko et al., 1998). In this work, the probe substrate concentrations of CYP 1A2, 2C9, 2B6, 2E1 and 3A4 (phenacetin, tolbutamide, (S)-mephenytoin, chlorzoxazone and testosterone) were 50, 150, 1000, 75 and 100 μM, respectively. The concentrations of vanillin/ethyl vanillin added to the incubations were ranged from 8 to 128 μM.

2.2.2. Determination of CYPs activities

To determine the production of acetaminophen, 4′-hydroxytolbutamide, nirvanol, 6-hydroxychlorzoxazone, and 6β-hydroxytestosterone, the residue was reconstituted with 100 μL of mobile phase and analyzed by previously published HPLC methods with slight modification (Jurica et al., 2010; Relling et al., 1990; Ko et al., 1998; Cao et al., 2008; Qiu et al., 2010). The separation was performed by using a Zorbax Eclipse XDB–C_{18} column (5 μm, 150 × 4.6 mm; Agilent, Waldbronn, Germany) fitted with a Zorbax C_{18} (5 μm, 12.5 × 4.6 mm; Agilent, Waldbronn, Germany) guard column. All metabolites were delivered at a flow rate of 0.8 mL/min. Other chromatographic conditions were displayed in Table 1.

The quality control (QC) samples of each metabolite were prepared at three concentration (low, middle and high) levels in HLM, and were used to evaluate the precision and relative recovery of the analytical methods established in the present work.

2.3. In vivo experiments

2.3.1. Animal treatments and preparation of liver microsomes

According to the acceptable daily intake (ADI) of vanillin and ethyl vanillin (0–10 and 0–3 mg/kg of body weight for humans, respectively) (JECFA, 2002), the low, medium and high oral dosages of rats were 3, 15 and 75 mg/kg vanillin/ethyl vanillin per day in the present work.

All animal procedures were conducted in accordance with guidelines for the care and the use of laboratory animals and approved by the Animal Care Committee of Hubei University. Male Wistar rats (180–200 g, Experimental Animal Center of Hubei province, Certificate Number: SCXK 2008-0005) of 6-week-old were housed for 7 days under SPF grade laboratory conditions (25 ± 2 °C, 60 ± 5% relative humidity, 12 h light–dark cycle) with free access to food and tap water throughout the experiments. A total of 21 rats were randomly divided into seven groups ($n = 3$/group) and receiving an oral dose of A: saline containing 0.5% CMC; B: vanillin 3 mg/kg/day; C: vanillin 15 mg/kg/day; D: vanillin 75 mg/kg/day; E: ethyl vanillin 3 mg/kg/day; F: ethyl vanillin 15 mg/kg/day; G: ethyl vanillin 75 mg/kg/day once a day for seven consecutive days. Rats were killed by cervical fracture and decapitation 1 h after the last treatment and livers were immediately removed and weighed. The minces of livers were washed with cold homogenization buffer (0.25 mol L^{-1}sucrose, 0.01 mol L^{-1}Tris–HCl, 1 mmol L^{-1} EDTA, pH 7.4) and used to prepare RLM according to the literature (Peng et al., 2009). RLM samples were stored at −80 °C until use. Protein concentration of RLM was determined by BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) standardized with bovine serum albumin.

2.3.2. Incubation in rat liver microsomes

All incubations were performed at 37 °C in a system containing rat microsomal protein (1 mg/mL for testosterone 6β-hydroxylation, *p*-nitrophenol hydroxylation and tolbutamide 4′-hydroxylation, 0.4 mg/mL for ethoxyresorufin *O*-deethylation) and a NADPH regenerating system (NADPH, 1 mM, $MgCl_2$, 5 mM) in a final volume of 200 μL (0.1 M potassium phosphate buffer, pH 7.4). After preincubation at 37 °C for 3 min, the incubation reaction was started by the addition of 1 mM NADPH in phosphate buffer. The duration of incubation was 10 min for testosterone and chlorzoxazone, 15 min for ethoxyresorufin and 45 min for tolbutamide. The reaction was stopped by cooling on ice and addition of 400 μL methanol (testosterone 6β-hydroxylation, tolbutamide 4′-hydroxylation and ethoxyresorufin *O*-deethylation) and 100 μL $HClO_4$ (0.6 M) (*p*-nitrophenol hydroxylation). Protein was precipitated by vortexing for 3 min and centrifugation at 12, 000 *g* for 10 min, then the supernatants (6β-hydroxytestosterone and 4′-hydroxytolbutamide) were evaporated (BioTron Ecospin 3180C LABCONCO, Korea) at 40 °C. The residues and supernatants were stored at −20 °C until analysis. The substrate concentrations used for CYP 1A2, 2C, 2E1 and 3A (ethoxyresorufin, tolbutamide, *p*-nitrophenol and testosterone) were 6.5, 250, 500 and 250 μM, respectively.

2.3.3. Determination of CYPs activities

The productions of 4′-hydroxytolbutamide, 4-nitrocatechol, resorufin and 6β-hydroxytestosterone were determined by previously published methods (Relling et al., 1990; Allis and Robinson, 1994; Sarich et al., 1999; Qiu et al., 2010) with slight modification.

HPLC methods for 6β-hydroxytestosterone and 4′-hydroxytolbutamide were the same as that of determinations of CYP3A4 and CYP 2C9 activities in HLM samples. Resorufin was determined by using a Multi-function Microplate Reader (TriStar LB 941, Berthold Technologies, Germany) with the excitation and emission wavelength at 530 and 586 nm. The absorbance of 4-nitrocatechol was determined at 530 nm.

2.4. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Two-way analysis of variance (two-way ANOVA) and the Student–Newman–Keuls multiple range test using Sigmastat TM (Jandel Co., SPSS Science, Chicago, IL, USA) were performed.

3. Results

3.1. Effect in vitro

3.1.1. Method validation

The retention time of acetaminophen, nirvanol, 4′-hydroxytolbutamide, 6-hydroxychlorzoxazone and 6β-hydroxytestosterone were 3.9 min, 13.5 min, 6.2 min, 5.1 min and 7.8 min, respectively. No significant interference was observed in the controls.

Table 1
HPLC methods for simultaneous determination of five cytochrome P450 (CYP) probe substrate metabolites.

CYPs	Metabolites	Mobile phase	UV wavelength (nm)
CYP1A2	Acetaminophen	23% methanol, 77% water (containing 10 mM potassium dihydrogen phosphate, pH = 4.5)	245
CYP2C9	4′-Hydroxytolbutamide	30% acetonitrile, 70% water (containing 0.1% acetic acid)	230
CYP2B6	Nirvanol	11.2% acetonitrile, 16.8% methanol, 72% water (containing 50 mM potassium dihydrogen phosphate, pH = 4.2)	204
CYP2E1	6-Hydroxychlorzoxazone	22% acetonitrile, 78% water	282
CYP3A4	6β-Hydroxytestosterone	55% methanol, 45% water	247