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Source / Izvornik: **Croatica Chemica Acta, 2019, 92, 115 - 123**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:142933

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Download date / Datum preuzimanja: **2024-04-27**

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ORIGINAL SCIENTIFIC PAPER

CROATICA CHEMICA ACTA

Croat. Chem. Acta **2019**, *92*(1), 115–123
Published online: July 3, 2019
DOI: 10.5562/cca3528

HKD 1926

Characterization of *O*-demethylations and Aromatic Hydroxylations Mediated by Cytochromes P450 in the Metabolism of Flavonoid Aglycons

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RECEIVED: June 1, 2019 ★ REVISED: June 18, 2019 ★ ACCEPTED: June 24, 2019

Abstract: One of the most important groups of metabolic enzymes is cytochrome P450 superfamily. These enzymes are important in terms of the catalytic diversity and the large number of xenobiotics that are detoxified or activated by converting to reactive metabolites. Flavonoids are xenobiotics to which humans are exposed through diet. Data on their oxidative metabolism mediated by cytochromes P450 are limited. The aim of this study was to determine the enzymatic kinetics of *O*-demethylation and aromatic hydroxylation of flavonoid aglycons on recombinant cytochrome P450 enzymes and human liver microsomes systems. The study was performed on ten flavonoids, namely 3,7-dihydroxyflavone, 7-hydroxyflavone, acacetin, apigenin, flavone, galangin, kaempferol, naringenin, sakuranetin, and tangeretin using liquid chromatography coupled with mass spectrometry and UV detector. Most relevant enzyme involved in metabolism of flavonoid aglycons is CYP1A2, and its catalytic effectiveness ranges from 0.5 to 2.9 × 10^6 M^{-1} min^{-1}. Having in mind high expression and involvement of CYP1A2 in metabolism of xenobiotics including drugs, and its intraindividual differences in expression and activity, potential of drug-flavonoid competitive interactions/inhibitions should be considered when consuming dietary supplement and foods rich in flavonoids.

Keywords: flavonoids, human liver microsomes, cytochromes P450, enzyme kinetics.

INTRODUCTION

METABOLISM is one of the ways by which human organisms protect themselves from foreign substances i.e. xenobiotics. Xenobiotics are usually biotransformed to more soluble metabolites susceptible to elimination.[1] One of the most important groups of metabolic enzymes is cytochrome P450 superfamily. These enzymes are important in terms of the catalytic diversity and the large number of xenobiotics that are detoxified or activated by converting to reactive metabolites.[1,2] Although present in all tissues, the highest concentration of cytochrome P450 involved in biotransformation of xenobiotics is found in liver endoplasmic reticulum (microsomes).

The liver microsomal cytochrome P450 have important role in determining the intensity and duration of the drug effect and also play a key role in the detoxification of xenobiotics.[2] The total number of cytochrome P450 substrates reaches several thousand. Such a wide spectrum of substrates is partly due to high number of cytochromes (57 in humans), and promiscuity in substrate selection. On one side, the cytochrome P450 enzyme system has a role in the metabolism of specific endogenous substrates such as fatty acids, steroid hormones and eicosanoids. While this is mainly attributed to mitochondrial cytochrome P450, microsomal cytochromes P450 have a main purpose to clean the body from ingested foreign substances such as terpenes, alkaloids, pyrolysis products, and other xenobiotics.[1–3]

Cytochromes P450 are major enzymes involved in drug metabolism. Metabolism of 75 % of all known small molecule drugs is mediated by cytochromes P450. Out of

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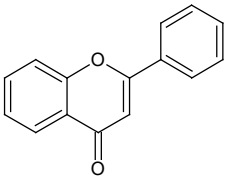
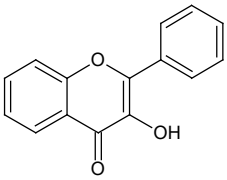
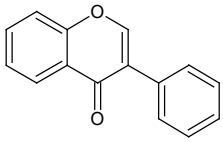
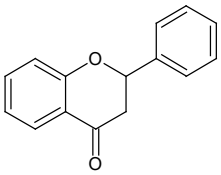
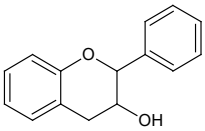
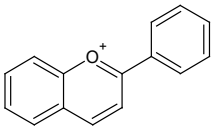
that number 90 % of oxidoreductions is mediated by CYP3A4/5, CYP2C9, CYP2C19, CYP2D6, CYP2C8, and CYP1A2.[4]

Flavonoids are xenobiotics to which we are exposed through diet. They represent low molecular weight polyphenol compounds characterized by the benzo-γ-pyrone (chromane) structure. Based on the modifications of benzo-γ-pyrone core, flavonoids are divided into six major subgroups (Table 1).[5–10]

Most of the beneficial effects of flavonoids on human health are attributed to their antioxidant and healing properties. Flavonoids exhibit unique cardio-protective effects due to their ability to inhibit lipid-mediated low density lipoproteins (LDL) oxidation. The protective role of diet rich in flavonoids has been demonstrated in several major prognostic studies. For example, it has been shown that a greater intake of flavonoids in the body lowers mortality due to coronary heart disease and lower incidence of myocardial infarction in older males. In postmenopausal women, the risk of coronary heart disease was reduced by 38 %.[9] Flavonoids can affect platelet aggregation at concentrations that can be achieved in the blood after consumption of foods rich in polyphenols.[11] Flavonoids can also interfere the results of

Table 1. Clasification, examples, structural characteristics and dietary sources of flavonoids. Flavonoids marked in bold have been analysed in this study

Class	Basic structure	Flavonoid	Substituents	Food sources
Flavones		**Acacetin** **Apigenin** Baikalein Chrysin Diosmetin Luteolin **Tangeretin**	5,7-OH-4'-OCH_3 5,7,4'-OH 5,6,7-OH 5,7-OH 5,7,3'-OH-4'-OCH_3 5,7,3',4'-OH 5,6,7,8,4'-OCH_3	fruits, parsley, celery, red peppers, tomato sauce, honey, propolis
Flavonols		**Galangin** **Kaempferol** Morin Myricetin Quercetin	3,5,7-OH 3,5,7,4'-OH 3,5,7,2',4'-OH 3,5,7,2',3',4'-OH 3,5,7,3',4'-OH	green leek, , kale, broccoli, salad, grapefruit, black tea, red onion, black wine, apple, berry fruits
Isoflavones		Genistein Diadzein Formononetin	5,7,4'-OH 7,4'-OH 7-OH-4'-OCH_3	peas, soya and other legumes
Flavanones		Eriodictyol Hesperetin Homoeriodictyol **Naringenin**	5,7,3',4'-OH 5,7,3'-OH-4'-OCH_3 5,7,4'-OH-3'-OCH_3 5,7,4'-OH	citruses (grapefruit, lemon, orange)
Flavanols		Catechin Epicatechin	3,5,7,3',4'-OH 3,5,7,3',4'-OH	tea (black and green), cocoa, chocolate, blueberry, black wine, hazelnut
Anthocyanidins		Cyanidin Apigenidin	3,5,7,3',4'-OH 5,7,4'-OH	coloured fruits, cherry, raspberry, strawberry

diagnostic functional assays (platelet aggregation induced by ristocetine and arachidonic acid), and show antitumoral, antioxidative and vasodilatory effects.[12–16] There are numerous other pharmacological effects of flavonoids described in the literature such as positive effects on capillary permeability and hypolipemic, antihypertensive, antimicrobial, antiviral, antiallergic, anti-inflammatory, antineoplastic, and hepatoprotective effects.[7,17]

Although the number of known flavonoids is extremely high, over 4000, most flavonoids have not been characterized in terms of biological effects, and knowledge about metabolism is known only for the most common dietary flavonoids.[18–33] In the previous study, screening of metabolic reactions mediated by cytochrome P450 was conducted on thirty flavonoid aglycons, most commonly found in medicinal plants and propolis.[34] Flavonoid aglycons are subject to *O*-demethylation and aromatic hydroxylation reactions, and their metabolism includes cytochrome P450, predominantly CYP1A2.[34] The aim of this study was to determine the enzymatic kinetics of *O*-demethylation and aromatic hydroxylation of flavonoid aglycons that have been shown to be susceptible to the metabolism mediated by human liver cytochromes P450 in the previously reported screening analysis.[34] The study was performed on ten flavonoids, namely 3,7-dihydroxyflavone, 7-hydroxyflavone, acacetin, apigenin, flavone, galangin, kaempferol, naringenin, sakuranetin, and tangeretin using the recombinant cytochrome P450 enzymes and human liver microsomes. Reactions were monitored using the liquid chromatography coupled with mass spectrometer and UV detector.

EXPERIMENTAL SECTION

Chemicals and Enzymes

Flavonoids used in this study (3,7-dihydroxyflavone, 7-hydroxyflavone, acacetin, apigenin, galangin, kaempferol, naringenin, pinocembrin, sakuranetin, and tangeretin) were acquired commercially from Sigma-Aldrich (St. Louis, MO, USA).

Human liver microsomes (HLM), and recombinant cytochromes P450 (CYP3A4, CYP2C9, CYP2C19, CYP2D6, CYP2C8, and CYP1A2) coexpressed with NADPH cytochrome P450 reductase and cytochrome b_5 in baculosomes were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and NADP disodium salt were purchased from Sigma Aldrich. Potassium phosphate (p.a.) and concentrated hydrochloric acid (p.a.) were purchased from Kemika d.d. (Zagreb, Croatia). Acetonitrile for chromatography was purchased from Merck KGaA (Darmstadt, Germany). Ultra-pure water was produced using Arium comfort combined water production system from Sartorius AG (Goettingen, Germany).

Incubations for Characterization of Metabolic Reactions Mediated by Human Liver Microsomes

Evaluation of enzyme kinetics was conducted using human liver microsomes (HLM) as source of enzyme. A range of concentrations from 2 to 200 µM flavonoid aglycons (2–800 µM in experiment with 3,7-dihydroxyflavone and 7-hydroxyflavone) was prepared; appropriate aliquots of 20 mM flavonoid methanolic solution were transferred to glass tubes and evaporated to dryness on a water bath equipped with mechanical shaking. After evaporation of the solvent, a 100 µL incubation mixture was prepared in each of the tubes by adding human liver microsomes (HLM, amount corresponding to 100 pmol cytochrome P450 enzyme), 50 mM potassium phosphate buffer pH 7.4 and ultra-pure water. Generating system containing glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase in a ratio of 100:50:2 (*V*/*V*/*V*) was used as a source of coenzyme, and its addition marked the beginning of the reaction (15 % volume in final incubation, *V*/*V*). In the negative control, 15 µL of ultra-pure water was added instead of generating system. After 15 minutes at 37 °C of incubation in water bath with mechanical shaking, the reaction was stopped by adding 60 µL of a mixture of acetonitrile and 25 % hydrochloric acid solution in a ratio of 5:1 (*V*/*V*). After stopping the reaction, each incubation mixture was centrifuged at 10,000 rpm for 10 minutes in a MiniSpin centrifuge (Eppendorf AG, Hamburg, Germany). The pure supernatant was transferred to the HPLC vial and analyzed on LC/MS/UVD.

Incubations for Characterization of Metabolic Reactions Mediated by Recombinant Cytochromes P450

Incubations with recombinant cytochromes P450 were conducted in the same manner as with the human liver microsomes. Instead of human liver microsomes, cytochromes P450 were used in incubations (10 pmol of CYP3A4 and CYP2D6 in experiment with 7-hydroxyflavone and CYP2C19 in the experiment with galangin; otherwise 3 pmol). Other incubation components were used in the same concentrations as stated above i.e. 50 mM potassium phosphate buffer pH 7.4, and 15 % volume of generating system. Volume of ulta-pure water was adjusted to final volume of 100 µL. Reaction was stopped and samples prepared for the LC/MS/UVD analysis, as described above.

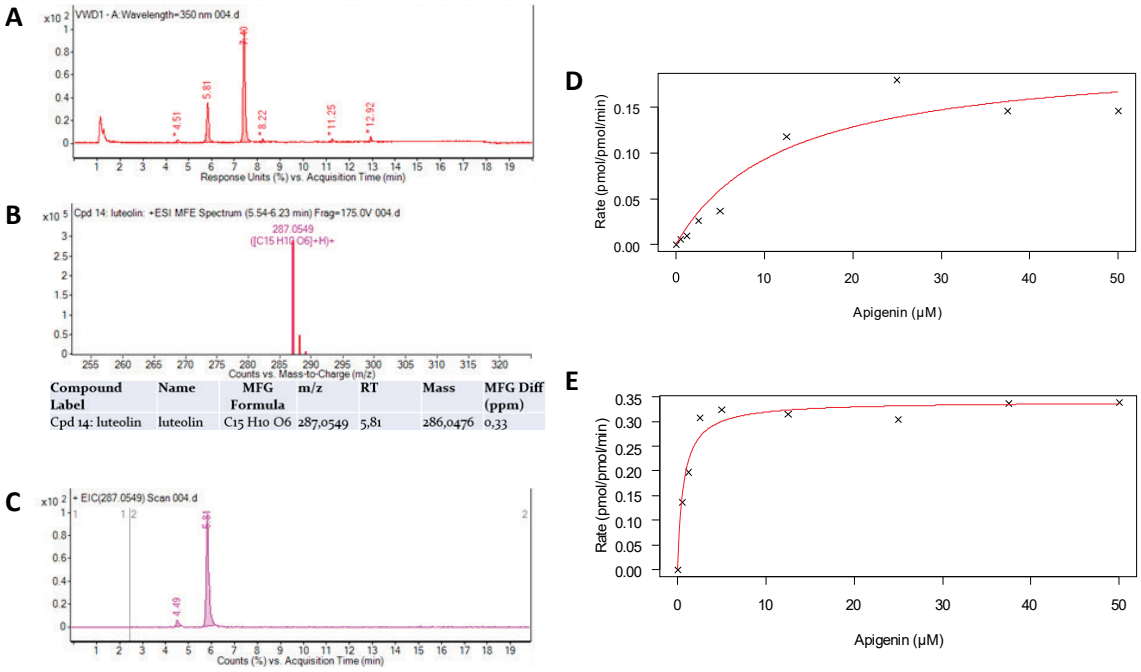
HPLC-MS-UV Analysis

High performance liquid chromatography (HPLC) coupled

with ultraviolet detector (UV) and mass spectrometer (MS) was performed on the Agilent 1200 RR (Agilent Technologies, Waldbronn, Germany) LC system coupled to Agilent 6530 Accurate Mass Q-TOF LC/MS Agilent Technologies (Waldbronn, Germany) using an electrospray ionization (ESI) interface. Separation was achieved on the Poroshell EC-C18 column (100 × 3.0 mm i.d., 2.7 µm particle size, Agilent). The temperature of column was set to 40 °C. The flow rate was 0.4 mL/min; mobile phase A consisted of water, methanol and formic acid at a ratio of 93:5:2 (*V*/*V*/*V*) while the ratio of the same components in mobile phase B was 3:95:2 (*V*/*V*/*V*). The gradient elution method was carried out according to the following timetable: 0 min, 40 % B; 14 min, 80 % B; 15 min, 80 % B; 16 min, 40 % B; 20 min, 40 % B. The UV detector was set at 350 nm. Mass spectrometry data collection was performed in MS High Resolution mode, ESI source was set to the positive ionization mode and mass spectra were recorded in *m*/*z* range of 100 to 1000. Operating conditions of the MS detector were as follows: drying gas flow 8 L/min at 40 psi nebulizer gas pressure and drying gas temperature at 200 °C, sheath gas flow was 11 L/min with sheath gas temperature set at 300 °C. The time-of-flight (TOF) analyzer was used with the following settings: the voltage of the fragment, skimmer and the octapol were 175 V, 65 V, and 750 V. All data were collected and processed using Agilent MassHunter Workstation Software Program (Agilent Technologies, United States). Identification of the metabolites was based on a comparison of exact molecular mass and time of metabolite retention relative to the comparative solution. Quantification of the detected metabolites was performed on the basis of the signals measured by the UV detector.

Determination of Enzyme Kinetic Parameters

All incubations were conducted in triplicate. The results were expressed as the amount of generated metabolite based on LC-MS-UVD analysis (*vide supra*). Based on these results reaction rates were determined and used for calculation of the major parameters of enzymatic kinetics based on the Michaelis-Menten equation. In addition to the parameters of Michaelis-Menten kinetics (Michaelis-Menten constants, K_m, and maximal rate of reaction, V_{max}), k_{cat} was calculated as V_{max}/enzyme concentration, as well as ratios k_{cat}/K_m (or V_{max}/K_m). Program R (The R Project for Statistical Computing, Vienna, Austria) was used for calculations of enzyme kinetic parameters.



Compound Label	Name	MFG Formula	m/z	RT	Mass	MFG Diff (ppm)
Cpd 14: luteolin	luteolin	C15 H10 O6	287,0549	5,81	286,0476	0,33

Figure 1. Methodology used for characterization of aromatic hydroxylation reaction. An example of apigenin, that is biotransformed into luteolin, is given. This is observed with human liver microsomes incubations analyzed by UVD (A), and identity of metabolite is confirmed by high resolution mass spectrometry (luteolin *m*/*z* = 287.0549, t_r = 5.8 min) (B). In apigenin incubations with CYP1A2 enzyme, apigenin is transformed to luetolin (t_r = 5.8 min) as shown by extracted ion chromatogram *m*/*z* = 287.0549 (C). Based on incubations with different concentrations of substrate, apigenin, Michealis-Menten equation paramters are calculated for the human liver microsomes incubations (k_{cat} = 0.2 min^{-1}, K_m = 12.4 µM) (D), as well as recombinant CYP1A2 enzyme (k_{cat} = 0.3 min^{-1}, K_m = 0.7 µM) (E).

RESULTS

In this study enzyme kinetics was determined for 3,7-dihydroxyflavone, 7-hydroxyflavone, acacetin, apigenin, flavone, galangin, kaempferol, naringenin, sakuranetin, and tangeretin. These were flavonoids that showed metabolite formation in the amount of at least 10 % (*m*/*m*) in comparison with the amount of substrate in the human liver microsomes screening experiments.[34] An example of methodology used to determine kinetic parameters is provided in Figure 1.

Enzyme kinetics was determined for 10 flavonoids on human liver microsomes and for 8 flavonoids it was also characterized on recombinant cytochromes P450. Summarized results are presented in Table 2.

Kinetic parameters were determined for the reaction of aromatic hydroxylation of 3,7-dihydroxyflavone in which 3,7,4'-trihydroxyflavone is generated. In case of human liver microsomes V_{max} was 5.9 ± 1.1 min^{-1} and K_m of 492 ± 163 μM was calculated. The catalytic effectiveness (k_{cat}/K_m) for this reaction was (0.012 ± 0.005) × 10^6 M^{-1} min^{-1}. While human liver microsomes catalyze the aromatic hydroxylation of the ring B, CYP1A2 catalyses the aromatic hydroxylation at the ring A with catalytic effectiveness of (1.5 ± 0.9) × 10^6 M^{-1} min^{-1}.

7-hydroxyflavone is hydroxylated at the postions 6, 8, or 4'; major metabolilic pathway being conversion to 6,7-dihydroxyflavone. In the experiments with the human liver microsomes V_{max} of the 6-hydroxylation was 1.9 ± 0.1 min^{-1} and K_m 43.5 ± 13.3 μM, catalytic effectiveness (k_{cat}/K_m) being (0.043 ± 0.014) × 10^6 M^{-1} min^{-1}. Reaction at the position 8 was ten times less effectively catalyzed (V_{max} = 0.40 ± 0.05 min^{-1}, K_m = 104 ± 37 μM, k_{cat}/K_m = (0.004 ± 0.002) × 10^6 M^{-1} min^{-1}). Overall catalytic effectiveness of aromatic hydroxylation of 7-hydroxyflavone mediated by human liver microsomes was (0.050 ± 0.016) × 10^6 M^{-1} min^{-1}. Major enzyme involved in hydroxylation at the position 6 was CYP3A4 in appropriate incubations with catalytic effectiveness of (0.054 ± 0.020) × 10^6 M^{-1} min^{-1}. Similar results were obtained for the reaction of aromatic hydroxylation at the position 8 with catalytic effectiveness of (0.052 ± 0.014) × 10^6 M^{-1} min^{-1}. Overall catalytic effectiveness of 7-hydroxyflavone aromatic hydroxylation mediated by CYP3A4 was (0.10 ± 0.03) × 10^6 M^{-1} min^{-1}. Interestingly, aromatic hydroxylation at the position 6 (but not at the position 8) was also mediated by CYP1A2 and CYP2D6. The catalytic effectiveness (k_{cat}/K_m) of CYP1A2 was (1.5 ± 0.3) × 10^6 M^{-1} min^{-1}, and for CYP2D6 this value was (0.06 ± 0.01) × 10^6 M^{-1} min^{-1}. While catalytic effectiveness of CYP1A2 is greater compared to CYP3A4, overall CYP3A4 contribution to the metabolism of 7-hydroxyflavone in human liver microsomes is more significant as CYP3A4 is more expressed compared to CYP1A2.

Acacetin undergoes demethylation to apigenin followed by aromatic hydroxylation to luteolin. Overall catalytic effectiveness in human liver microsomes system for both reactions was (0.06 ± 0.02) × 10^6 M^{-1} min^{-1}. Major enzyme involved in this metabolic conversion, both demethylation and aromatic hydroxylation, was CYP1A2 having the catalytic effectiveness of (2.9 ± 1.5) × 10^6 M^{-1} min^{-1}. Interestingly, CYP2D6 only catalyzed demethylation reaction with the catalytic effectiveness of (0.16 ± 0.05) × 10^6 M^{-1} min^{-1}.

As shown on Figure 1, apigenin undergoes aromatic hydroxylation to luteolin and as expected from experiments with acacetin this conversion is mediated by CYP1A2. The catalytic effectiveness determined on human liver microsomes was (0.017 ± 0.009) × 10^6 M^{-1} min^{-1}, and in case of recombinant CYP1A2 this value was (0.5 ± 0.1) × 10^6 M^{-1} min^{-1}.

In case of flavone enzyme kinetic parameters could not be determined, as the formation of flavone metabolite was linearly dependent to the concentration used in this experiment (higher concentration of flavone could not be used due to solubility issue in physiological water media). While V_{max} and K_m values could not be determined, catalytic effectiveness could be extrapolated as a slope value of linear regression (under low concentrations of substrate rate of metabolite production is linearly dependent to substrate concentration).[35] Thus, catalytic effectiveness of 4'-hydroxylation of flavone catalyzed by human liver microsomes was (0.0015 ± 0.0001) × 10^6 M^{-1} min^{-1}. Minor products of aromatic hydroxylation are also generated at the positions 6 and 7, and overall catalytic effectiveness was (0.0028 ± 0.0002) × 10^6 M^{-1} min^{-1}.

Galangin undergoes sequential hydroxylation to quercetin through kaempferol. In case of human liver microsomes, overall catalytic effectiveness of this biotransformation was (0.11 ± 0.06) × 10^6 M^{-1} min^{-1}. Biotransformation of galangin to kaempferol is catalyzed by CYP2C19 with a catalytic effectiveness of (0.004 ± 0.001) × 10^6 M^{-1} min^{-1}.

Aromatic hydroxylation of kaempferol to quercetin was individually studied. In human liver microsomes catalytic effectiveness was (0.024 ± 0.010) × 10^6 M^{-1} min^{-1}, as determined in human liver microsomes system.

Naringenin is metabolized to eriodictyol in the reaction of aromatic hydroxylation catalyzed by cytochrome P450 HLM in appropriate incubations. The catalytic effectiveness constant was (0.025 ± 0.009) × 10^6 M^{-1} min^{-1}. CYP1A2 is major cytochrome P450 involved in this reaction with catalytic effectiveness of (0.003 ± 0.002) × 10^6 M^{-1} min^{-1}. Since this is a slow reaction characterized by low efficiency, a very small amount of metabolite is formed in the incubations and the error of measurement is more pronounced in the results of the kinetic parameters.

Table 2. Results of enzyme kinetics of metabolic reactions mediated by cytochromes P450 observed in human liver microsomes (HLM), and determined on the HLM and recombinant cytochromes P450.

Substrate	Product	Reaction	Enzyme system	k_{cat} / min^{-1}	K_m / μM	(k_{cat}/K_m) / 10^{-6} min^{-1} M^{-1}
3,7-dihydroxyflavone	3,7,4'-trihydroxyflavone	hydroxylation	HLM	5.9 ± 1.1	492 ± 163	0.012 ± 0.005
	3,6,7-trihydroxyflavone or 3,7,8-trihydroxyflavone	hydroxylation	CYP1A2	6.1 ± 0.7	4.2 ± 2.6	1.5 ± 0.9
7-hydroxyflavone	6,7-dihydroxyflavone + 7,8-dihydroxyflavone + 7,4'-dihydroxyflavone	hydroxylation	HLM	2.7 ± 0.2	54.9 ± 16.7	0.050 ± 0.016
	6,7-dihydroxyflavone	hydroxylation	HLM	1.9 ± 0.1	43.5 ± 13.3	0.043 ± 0.014
	7,8-dihydroxyflavone	hydroxylation	HLM	0.40 ± 0.05	104 ± 37	0.004 ± 0.002
	7,4'-dihydroxyflavone	hydroxylation	HLM	0.50 ± 0.04	82.0 ± 21.7	0.006 ± 0.002
	6,7-dihydroxyflavone + 7,8-dihydroxyflavone	hydroxylation	CYP3A4	3.9 ± 0.3	37.8 ± 10.3	0.10 ± 0.03
	6,7-dihydroxyflavone	hydroxylation	CYP3A4	2.6 ± 0.3	47.9 ± 16.4	0.054 ± 0.020
	7,8-dihydroxyflavone	hydroxylation	CYP3A4	1.4 ± 0.1	26.2 ± 6.6	0.052 ± 0.014
	6,7-dihydroxyflavone	hydroxylation	CYP2D6	1.60 ± 0.08	26.0 ± 4.8	0.06 ± 0.01
	6,7-dihydroxyflavone	hydroxylation	CYP1A2	14.2 ± 0.7	9.7 ± 2.0	1.5 ± 0.3
acacetin	apigenin + luteolin	demethylation + hydroxylation	HLM	0.62 ± 0.07	9.5 ± 3.3	0.06 ± 0.02
	apigenin + luteolin	demethylation + hydroxylation	CYP1A2	3.8 ± 0.4	1.3 ± 0.6	2.9 ± 1.5
	apigenin	demethylation	CYP2D6	3.7 ± 0.5	22.7 ± 7.0	0.16 ± 0.05
apigenin	luteolin	hydroxylation	HLM	0.20 ± 0.03	12.4 ± 6.0	0.017 ± 0.009
	luteolin	hydroxylation	CYP1A2	0.30 ± 0.01	0.7 ± 0.1	0.5 ± 0.1
flavone	4'-hydroxyflavone + 6-hydroxyflavone + 7-hydroxyflavone	hydroxylation	HLM	/	/	0.0028 ± 0.0002
	4'-hydroxyflavone	hydroxylation	HLM	/	/	0.0015 ± 0.0001
galangin	kaempferol + quercetin	hydroxylation	HLM	3.3 ± 0.5	31.6 ± 16.0	0.11 ± 0.06
	kaempferol	hydroxylation	CYP2C19	0.022 ± 0.003	5.4 ± 1.4	0.004 ± 0.001
kaempferol	quercetin	hydroxylation	HLM	0.082 ± 0.009	3.4 ± 1.5	0.024 ± 0.010
naringenin	eriodyctiol	hydroxylation	HLM	1.1 ± 0.2	45.2 ± 14.9	0.025 ± 0.009
	eriodyctiol	hydroxylation	CYP1A2	0.021 ± 0.003	7.3 ± 5.6	0.003 ± 0.002
sakuranetin	5,3',4'-trihydroxy-7-methoxyflavanone + naringenin + eriodiktiol	hydroxylation + demethylation + hydroxylation	HLM	5.4 ± 1.0	293 ± 82	0.019 ± 0.006
	naringenin + eriodiktiol	demethylation + hydroxylation	HLM	3.1 ± 0.3	152 ± 31	0.020 ± 0.005
	5,3',4'-trihydroxy-7-methoxyflavanone	hydroxylation	HLM	/	/	0.0022 ± 0.0002
	5,3',4'-trihydroxy-7-methoxyflavanone	hydroxylation	CYP3A4	2.2 ± 0.4	39.8 ± 20.1	0.06 ± 0.03
	5,3',4'-trihydroxy-7-methoxyflavanone	hydroxylation	CYP1A2	8.3 ± 1.1	12.2 ± 6.8	0.7 ± 0.4
tangeretin	X-demethylated product (ring A)	demethylation	HLM	2.2 ± 0.3	97.3 ± 29.2	0.023 ± 0.008
	4'-hydroxy-5,6,7,8-tetramethoxyflavone + 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone	demethylation + hydroxylation	CYP1A2	8.7 ± 0.5	9.1 ± 2.3	1.0 ± 0.2
	4'-hydroxy-5,6,7,8-tetramethoxyflavone	demethylation	CYP1A2	6.2 ± 0.3	12.9 ± 2.6	0.5 ± 0.1
	3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone	hydroxylation (demethylation)	CYP1A2	2.4 ± 0.1	4.5 ± 1.0	0.5 ± 0.1
	X-demethylated product (ring A)	demethylation	CYP3A4	13.8 ± 0.8	65.4 ± 9.9	0.21 ± 0.03
	4'-hydroxy-5,6,7,8-tetramethoxyflavone	demethylation	CYP3A4	6.4 ± 0.4	59.3 ± 9.0	0.11 ± 0.02
	4'-hydroxy-5,6,7,8-tetramethoxyflavone	demethylation	CYP2D6	/	/	0.0058 ± 0.0001

Therefore, the obtained results can be taken as a rough estimate of the kinetics for this reaction.

Sakuranetin undergoes sequential biotransformation, first demethylation to naringenin, then aromatic hydroxylation to eriodictyol. At the same time, minor product of direct aromatic hydroxylation of sakuranetin is formed i.e. 5,3',4'-trihydroxy-7-methoxyflavanone. Both metabolic pathways are observed in incubations with human liver microsomes and sequential biotransformation is 10 times more catalytic effective ((0.020 ± 0.005) × 10^6 M^{-1} min^{-1}) when compared to hydroxylation at the positon 3' ((0.0022 ± 0.0002) × 10^6 M^{-1} min^{-1}). Interestingly, only minor reaction was directly linked with cytochromes P450 used in this study i.e. CYP3A4 and CYP1A2 for which catalytic effectiveness constants were (0.06 ± 0.03) × 10^6 M^{-1} min^{-1} and (0.7 ± 0.4) × 10^6 M^{-1} min^{-1}, respectively.

Because of the interferences of metabolites on the UV detector in enzyme kinetics incubations with human liver microsomes, it was not possible to follow the main metabolic pathway of tangeretin in which tangeretin is demethylated to 4'-hydroxy-5,6,7,8-tetramethoxyflavone and then hydroxylated to 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone. However, these interferences were not present in incubations with recombinant enzymes. The most important enzyme for this metabolic pathway was CYP1A2 with catalytic effectiveness of (1.0 ± 0.2) × 10^6 M^{-1} min^{-1}. Kinetic parameters were determined on HLM for the reaction of single demethylation of tangeretin on ring A for the metabolite at t_R = 10.85 min. The catalytic effectiveness constant for this reaction was (0.023 ± 0.008) × 10^6 M^{-1} min^{-1}. This reaction was mediated by CYP3A4 (k_{cat}/K_m = (0.21 ± 0.03) × 10^6 M^{-1} min^{-1}). Demethylation of tangeretin at the position 4' is catalyzed by CYP3A4 (k_{cat}/K_m = (0.11 ± 0.02) × 10^6 M^{-1} min^{-1}), and to a lesser extent with CYP2D6 (k_{cat}/K_m = (0.0058 ± 0.0001) × 10^6 M^{-1} min^{-1}).

DISCUSSION

In this study we have characterized kinetic parameters of *O*-demethylations and aromatic hydroxylations to which flavonoid aglycons are susceptible. The range of values for rate constant k_{cat} was 0.021 to 14.2 min^{-1}. The Michaelis-Menten constants, K_m, was obtained in the range of 0.7 to 492 μM, and calculated catalytic effectiveness, k_{cat}/K_m, was in the range from 0.0015 to 2.9 × 10^6 M^{-1} min^{-1}. The range of results obtained for all kinetic parameters is very wide, and within a three-orders of magnitude. If we have in mind the structural homogeneity of the analyzed compounds, these results indicate a relatively high selectivity of human liver cytochrome P450 to certain substrates from this group of compounds. This conclusion is supported by the fact that only a few recombinant cytochromes P450 (CYP1A2, CYPC19, CYP2D6 and CYP3A4) catalyze the observed metabolic reactions of the analyzed flavonoids.

The enzyme that is most efficient is CYP1A2. With the exception of metabolic conversion of naringenin to eriodictyol, CYP1A2 catalytic effectiveness, k_{cat}/K_m, was in the range from 0.5 to 2.9 × 10^6 M^{-1} min^{-1}, indicating the high catalytic specificity or efficacy of this cytochrome P450 in flavonoid metabolism. This result is in agreement with published data on the metabolism of flavonoids mediated by cytochromes P450, in which most authors of the studies concluded that CYP1A2 plays a major role in the oxidative metabolism of flavonoids in *in vitro* conditions.[19,20,22,23,25,26,28,30,32]

Given the low concentrations generally achieved by oral intake of flavonoids, a very important kinetic parameter to consider is the K_m value. This is especially important in sense that flavonoids are known to have very low solubility in water and, most importantly, poor bioavailability after oral administration. Consequently, the usual concentrations achieved in the blood after the flavonoids consumption from different sources are nanomolar to micromolar, with large interindividual differences, and most often do not exceed 10 μM values.[36] Flavonoids for which the low K_m value has been determined have a higher chance of being metabolized *in vivo* after their oral administration compared to those having higher catalytic conversion rate (k_{cat}) and high K_m. In that sense, flavonoids that have higher potential of competitive interactions with other xenobiotics are: apigenin, acacetin, kaempferol, 3,7-dihydroxyflavone and galangin; all having K_m values bellow 10 μM (Table 2).

Out of ten analyzed flavonoids, seven of them are susceptible to the metabolism mediated by CYP1A2 (Table 2), which further confirms the hypothesis that this cytochrome P450 is the most important for the metabolism of flavonoids in humans. However, for a better estimate of the possible clinical significance of these metabolic reactions, it is not enough to consider only the K_m parameter. This parameter needs to be put in relation to the rate constant (k_{cat}) to provide k_{cat}/K_m ratio known as catalytic effectiveness. E.g. kaempferol, galagnin, and naringenin, have low K_m values, but given the relatively low rate of catalyst constant, it is expected that the extent of these reactions will not be so significant.

The range of the k_{cat}/K_m values of the studied reactions catalyzed by human liver microsomes was (0.0015 – 2.9) × 10^6 M^{-1} min^{-1}. It has been previously pointed out that the values of k_{cat}/K_m ratio catalyzed by CYP1A2 enzymes ranged from 0.003 to 2.9 × 10^6 M^{-1} min^{-1}. Therefore, it is not surprising that reactions with the highest catalytic efficiency are catalyzed by CYP1A2. Tangeretin demethylation reactions catalyzed by CYP3A4

and CYP2D6 are only secondary metabolic pathways of this flavonoid, while major metabolic pathways are catalyzed by CYP1A2. Considering that CYP1A2 is one of the most commonly found cytochromes P450 in the liver and one of the most important enzyme involved in drug metabolism,[4] potential of interactions of drug-flavonoid interaction cannot be ruled out, especially in case of acacetin, 3,7-dihydroxyflavone, 7-hydroxyflavone, tangeretin, sakuranetin, apigenin, and galangin, with drugs that are primarily metabolized by CYP1A2 such as clozapine, duloxetine, fluvoxamine, haloperidol, imipramine, naproxen, olanzapine, zolmitriptan, etc.[15] When considering these interactions, it is important to keep in mind the great interindividual differences observed in CYP1A2 expression and activity, which ranges up to 40 folds.[4,37]

It has already been pointed out that very little information on the metabolism of flavonoids mediated by cytochrome P450 is available in the literature. Data on kinetics of flavonoid metabolism is even less available. Bursztyka *et al.* and Hu *et al.* have studied kinetics of aromatic hydroxylation of genistein mediated by human liver microsomes and CYP1A2.[30,31] Wen and Walle studied the kinetics of demethylation of 5,7-dimethoxyflavone and 3',4'-dimethoxyflavone mediated by HLM.[38] Gradolatto *et al.* investigated the kinetics of aromatic hydroxylation of apigenin and the formation of luteolin in the incubation with rat liver microsomes.[21] Galangin metabolism and the kinetics of its conversion to kaempferol mediated by HLM were studied by Otake and Walle, and Otake *et al*.[26,39] The latter group of authors also studied the same reaction using CYP1A1, CYP1A2 and CYP2C9 enzymes. In all of these studies there is a common problem that makes it difficult to interpret the results of the enzyme kinetics. All incubations based on which the kinetic parameters were determined, lack the exact molar concentrations of the cytochrome P450 enzyme used in incubations. Instead of the molar concentration, the authors of the afore-mentioned studies used the protein mass unit in the incubations to express the results of these kinetic parameters. In these cases, k_{cat}/K_m constants included amounts of protein expressed in mg. This parameter is termed intrinsic clearance, which, according to Guengerich, is not good for comparison as it causes confusion.[16] The term "clearance" has a specific meaning in the *in vivo* pharmacokinetics.

In the literature only two studies have reported the kinetics of the metabolic reactions of flavonoids in which the actual parameters of their enzymatic kinetics k_{cat} and k_{cat}/K_m were comparable to the results obtained in this study. Androutsopulos *et al.* studied kinetics of diosmetin metabolism and its conversion into luteolin by the CYP1A1, CYP1A2 and CYP1B1 enzymes.[22] The kinetic parameters of this reaction were determined and the catalytic effectiveness constant was 8.1×10^6 M^{-1} min^{-1}. However, our previous results do not indicate extensive metabolism of diosmetin which could be due to different enzyme sources used in these studies. Another extensive study of the ipriflavone metabolism was conducted by Moon *et al.* using recombinant CYP1A2, CYP3A4, CYP2C9, and CYP2C19.[8] Values of catalytic effectiveness were in the range of $(0.02 - 0.22) \times 10^6$ M^{-1} min^{-1}, and these are comparable to the results we obtained in our study.

CONCLUSION

Kinetics of *O*-demethylation and aromatic hydroxylation reactions of ten flavonoids were characterized on human liver microsomes cytochrome P450 system. For eight of them characterization was also conducted on recombinant cytochromes P450. Most relevant enzyme involved in metabolism of flavonoid aglycons is CYP1A2, and it catalyzes biotransformation of acacetin, 3,7-dihydroxyflavone, 7-hydroxyflavone, tangeretin, sakuranetin, apigenin and galangin (aligned according to the catalytic effectiveness). Having in mind high expression and involvement of CYP1A2 in metabolism of xenobiotics including drugs, and its intraindividual differences in expression, potential of drug-flavonoid competitive interactions/inhibitions should be considered when consuming dietary supplement and foods rich in flavonoids.

Acknowledgment. This research was supported by the Croatian Science Foundation under the project UIP-2014-09-5704 (M. B.).

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