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Short Communication

Sulfation of selected mono-hydroxyflavones by sulfotransferases *in vitro*: a species and gender comparison

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Abstract

Objectives Sulfation via sulfotransferases is an important metabolic pathway contributing to the low bioavailability of flavonoids. This study aims to characterize the sulfation of mono-hydroxyflavones (MHFs) to obtain useful information on structure-metabolizing relationships in animal species and gender differences.

Methods Three representative MHFs, namely, 7-, 6- and 4′-MHF, were studied by incubating each MHF at different concentrations with various liver S9 fractions (mouse, rat, dog and human).

Key findings One mono-sulfate was identified for each MHF. 7-MHF and 4′-MHF usually have greater sulfations than 6-MHF. Regardless of whether the S9 fraction came from a male or female, there was a difference in sulfation in the species observed for all MHFs; the highest activity of sulfotransferases was in dog S9. Furthermore, gender differences affect sulfation of MHFs significantly. In rats, all sulfations for the three MHFs were higher in males than that in females while the opposite was observed in mice.

Conclusions Regiospecific, species and gender dependence exist in the sulfonation of all selected MHFs.

Keywords gender; mono-hydroxyflavones; regiospecific; species; sulfotransferases

Introduction

Dietary flavonoids include a broad range of compounds that are known to have a variety of claimed healthy benefits such as anti-cancer, antioxidant, anti-aging and cholesterol-lowering.[1] However, there is still no flavonoid therapeutic agent in the current market because of the poor bioavailability of these agents.[2] Glucuronidation and sulfation in the liver and intestine are two of the major barriers to the oral bioavailability.[3,4] The glucuronidation of flavonoids catalysed by UDP-glucuronosyltransferases has been extensively demonstrated in the literature.[5,6] In contrast, only a few studies have involved the sulfation of flavonoids.[7,8]

The glucuronidation of isoflavones, such as daidezein, glycitein and prunetin, as well as flavones such as mono- and di-hydroxyflavones, is significantly affected by their structure, especially by the hydroxyl group position.[9,10] However, the relationship between the sulfation of flavonoids and their structures is not known. Additionally, flavone is one of the main subgroups of flavonoids, and the sulfation characteristics of three selected MHFs with similar and simple structural properties, namely, 7-, 6- and 4′-MHF (Figure 1), were investigated.

Many earlier studies indicated that the main sites of flavonoid metabolism are in the liver and intestine.[11] Therefore, liver S9 fractions were chosen for this study. Reports state that glucuronidation and sulfation of 7-hydroxycoumarin in liver matrices from humans, dogs, monkeys, rats and mice are different.[12] Previous studies also indicated that gender may influence the activity of some conjugating enzymes, mainly due to the steroid hormone levels and metabolizing-enzyme levels including glucuronosyltransferase and sulfotransferases.[13] Punt *et al*.[14] reported that sulfation was about 30 times more efficient by male rat liver S9 than by human liver S9, whereas the catalytic efficiency by male mouse and human liver S9 was about the same, which agrees with our results. Our study also indicated that the

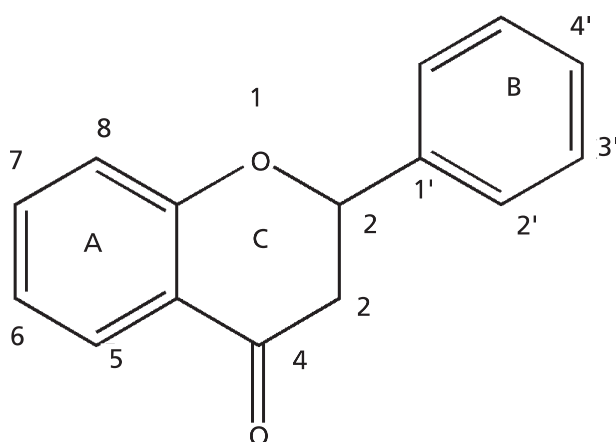

Figure 1 Structures of model flavones used in this study. Shown in the scheme are structures of aglycone forms of hydroxyflavone analogues. Three MHFs were chosen in the experiment. Conjugated forms of flavones with the sulfated metabolites may be attached to the 7-, 6- and 4′-positions.

species differences affected the glucuronidation of emodin *in vitro* and *in vivo*.[15] As a result, mouse, rat, dog and human liver S9 fractions were used in the present study.

Published literature also shows that gender-divergent sulfotransferases (SULTs) are mostly female-predominant and only SULT1c1 is male-dominant.[16] These studies indicate that there may be difference in the sulfation for the three mono-hydroxyflavones we are interested in. So we also chose pooled female and male liver S9 fractions to investigate the gender difference.

This study has two major objectives: (i) to determine how species and gender affect sulfation by incubating the flavones with liver S9 fractions and cofactors, and (ii) to investigate whether different structures and substrate concentrations of flavones influence the sulfation. This present investigation is novel and will shed light on the preclinical study of the sulfation of flavones in different genders and species systematically.

Materials and Methods

7-, 6-, and 4′-MHF (purity > 98%), adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS) and sulfatase were purchased from Sigma-Aldrich (St Louis, USA). CD-1 pooled female and male mouse liver S9, Fischer 344 pooled female and male rat liver S9, pooled female and male dog liver S9, pooled mouse liver S9, pooled rat liver S9, pooled dog liver S9 and pooled human (8 female, 16 male) liver S9 were purchased from BD Bioscience (Woburn, USA). All other materials were of analytical grade or higher.

The sulfation of flavones by liver S9 fractions was essentially the same as described previously[11] with minor modifications; there was no NADPH (the coenzyme of P450), UDPGA (uridine diphosphoglucuronic acid, the glucuronic acid donator of UGTs) or conjugating donators of other metabolizing enzymes in the liver S9 sulfation system. Briefly, the incubation mixture contained S9 fractions (final concentration = 0.5 mg/ml in a 50 mM potassium phosphate buffer, pH = 7.4), PAPS (final concentration = 100 μM) and different concentrations of flavones (final volume 200 μl). The mixed reaction system was incubated at 37°C in a shaking (50 rev/min) water bath for 30–90 min. Two controls were incubated in the absence of PAPS or without liver S9 fractions. The reactions were terminated by adding 100 μl solution of 94% acetonitrile/6% glacial acetic acid containing 50 μM internal standard testosterone. The supernatants after centrifugation were subjected to assay using ultra-performance liquid chromatography (UPLC).

The UPLC conditions for analysing the MHFs and their conjugates, and the elution program, were based on a published method.[9] The main working parameters for the mass spectrometers were: capillary voltage of 3.0 kV, cone voltage of 35 V, ion source temperature of 110°C, and minor adjustments were then made for each MHF. Mono-sulfates were identified by MS and MS2 full scan modes. An MHF standard curve was used for the quantification of MHF sulfate by using a conversion factor (*K*) because of the lack of MHF sulfate standards. This was done using similar methods to those used previously in our laboratory for flavonoids.[9]

All the data were analysed using Kruskal–Wallis test or one-way analysis of variance with Dunnett's multiple comparison (post-hoc) tests. $P < 0.05$ denoted significant difference.

Results and Discussion

Each of the three flavones has one metabolite, which was identified as the sulfate. For all the MHFs, the pseudomolecular ion $[M + H]^+$ of sulfates were at m/z 319, which was 80 Da higher (characteristic of the sulfuric acid addition) than that of MHFs, in which the pseudomolecular ion appeared at m/z 239. Based on these data, they were identified as the sulfates of MHFs (Table 1).

The species effects were compared and there was significant difference in species. Sulfation rates of the three MHFs at 5 μM, 10 μM and 40 μM concentrations were measured using mouse, rat, dog and human pooled liver S9 fractions. For 7-MHF, the rank orders of the sulfation rate (nmol/min/mg) (Table 2) at 5 μM, 10 μM and 40 μM were dog (0.1558 ± 0.0093, 0.1997 ± 0.0064 and 0.1475 ± 0.0040) > rat (0.1260 ± 0.0013, 0.1180 ± 0.0013 and 0.1376 ± 0.0026) > human (0.0810 ± 0.0026, 0.0577 ± 0.0011 and 0.0375 ± 0.0010) > mouse (0.0220 ± 0.0001 0.0230 ± 0.0002 and 0.0235 ± 0.0008) (Table 2). For 6-MHF, the sulfation rates in dog (all about 0.0275–0.0459 nmol/min/mg) liver S9 fractions were much faster than other species, followed by rat, human and mouse at all three substrate concentrations. However, for 4′-MHF, the trend was different (Table 2), the rate (nmol/min/mg) of 4′-O-S formation was always the highest in dog liver S9 at three concentrations, followed by mouse, rat and human at 5 μM. At 10 μM and 40 μM, the order was dog > rat > mouse > human ($P < 0.05$, Kruskal–Wallis

Table 1 UPLC and LC/MS/MS characteristics and the conversion factors of the selected mono-hydroxyflavones

Selected MHFs	Sulfates	Chromatographic retention time (min)		Characteristic ions in LC/MS/MS		*K* (mean ± SD)
		M	MHF	[M + H]	[M-S + H]	
7-MHF	7-O-S	1.82	2.50	319	239	0.98 ± 0.04
6-MHF	6-O-S	1.79	2.66	319	239	1.59 ± 0.04
4′-MHF	4′-O-S	1.84	2.65	319	239	1.21 ± 0.05

K, conversion factor (mean ± SD). Conversion factors were determined separately at three different concentrations.

Table 2 Species-dependent sulfation rates of three flavones (7-MHF, 6-MHF and 4′-MHF) in liver S9 fractions prepared from four different species (mouse, rat, dog, human)

Sulfation rates (nmol/min/mg, mean ± SD)		Mouse	Rat	Dog	Human
7-MHF	5 μM	0.0220 ± 0.0001$^{\#\blacktriangle}$	0.1260 ± 0.0013$^{*\blacktriangle}$	0.1558 ± 0.0093$^{*\#}$	0.0810 ± 0.0026$^{*\#\blacktriangle}$
	10 μM	0.0230 ± 0.0002$^{\#\blacktriangle}$	0.1180 ± 0.0013$^{*\blacktriangle}$	0.1997 ± 0.0064$^{*\#}$	0.0577 ± 0.0011$^{*\#\blacktriangle}$
	40 μM	0.0235 ± 0.0008$^{\#\blacktriangle}$	0.1376 ± 0.0026$^{*\blacktriangle}$	0.1475 ± 0.0040$^{*\#}$	0.0375 ± 0.0010$^{*\#\blacktriangle}$
6-MHF	5 μM	0.0007 ± 0.0001$^{\#\blacktriangle}$	0.0060 ± 0.0004$^{*\blacktriangle}$	0.0275 ± 0.0005$^{*\#}$	0.0021 ± 0.0001$^{*\#\blacktriangle}$
	10 μM	0.0007 ± 0.0001$^{\#\blacktriangle}$	0.0062 ± 0.0001$^{*\blacktriangle}$	0.0293 ± 0.0010$^{*\#}$	0.0029 ± 0.0001$^{*\#\blacktriangle}$
	40 μM	0.0010 ± 0.0001$^{\#\blacktriangle}$	0.0123 ± 0.0008$^{*\blacktriangle}$	0.0459 ± 0.0005$^{*\#}$	0.0067 ± 0.0004$^{*\#\blacktriangle}$
4′-MHF	5 μM	0.0457 ± 0.0045$^{\#\blacktriangle}$	0.0405 ± 0.0002$^{*\blacktriangle}$	0.1458 ± 0.0127$^{*\#}$	0.0384 ± 0.0062$^{*\#\blacktriangle}$
	10 μM	0.0366 ± 0.0026$^{\#\blacktriangle}$	0.0418 ± 0.0021$^{*\blacktriangle}$	0.1467 ± 0.0031$^{*\#}$	0.0302 ± 0.0027$^{*\#\blacktriangle}$
	40 μM	0.0312 ± 0.0006$^{\#\blacktriangle}$	0.0358 ± 0.0011$^{*\blacktriangle}$	0.1388 ± 0.0069$^{*\#}$	0.0203 ± 0.0008$^{*\#\blacktriangle}$

Three different concentrations (5 μM, 10 μM, 40 μM) were used in the experiment ($n = 3$). Sulfation rates were calculated as nmol/min/mg protein. *$P < 0.05$ vs mouse, #$P < 0.05$ vs rat, $^{\blacktriangle}P < 0.05$ vs dog ($P < 0.05$, Kruskall–Wallis)*$P < 0.05$ vs mouse, #$P < 0.05$ vs rat, $^{\blacktriangle}P < 0.05$ vs dog (Kruskall–Wallis test).

test). The above results clearly showed that the metabolic rate in dog liver S9 fractions was the fastest among the four selected liver S9 fractions for all three MHFs at three substrate concentrations.

Moreover, the species dependence was changed according to the substitutional positions of the hydroxyl group and the concentration of drug compounds. The sulfation rate of 7-MHF and 4′-MHF in rat, mouse and human liver S9 fractions was also observed to be much faster than that of 6-MHF. For 7-MHF, the rate was about 5- to 20-fold faster than that of 6-MHF and for 4′-MHF, the rate was about 5- to 10-fold faster than 6-MHF in rat, mouse, and human liver S9 fractions. Wang *et al.*[17] reported that the Km for 7-hydroxycoumarin sulfate formation showed no significant difference among humans, monkeys and rats (approximately 3 μM). Also, Wang *et al.*[18] reported that liver S9 samples from dogs, monkeys and humans had higher activity for formation of O-demethylapixaban sulfate than those from mice, rats and rabbits. Our data showed significant difference. The explanation could be that the sulfation of the substrates is regiospecific.

Published literatures showed that metabolism in different gender were respect to CYP (cytochrome P450) activity. Lofgren *et al.*[19] reported that female mice generally had a higher metabolism of bufuralol 1′-hydroxylation and dextromethorphan O-demethylation (human markers for CYP2D activity). Besides, Hernandez *et al.*[20] also reported greater CYP2B induction in female mice than male. Gender differences in humans were also observed with female human liver microsome (HLM) possessing greater activity than male HLM.[21]The gender effects on the rates of sulfation were also compared in our study. In rat liver S9 fractions (Table 3), the sulfation rates of all MHFs at three concentrations were much higher in males than in females ($P < 0.05$, Kruskall–Wallis test). However, in mouse liver S9 fractions (Table 3), the sulfation rates of 4′-MHF and 6-MHF at all three concentrations were much higher in females than in males, while for 7-MHF, statistically significant differences between genders were present only at 10 μM and 40 μM ($P < 0.05$). In dog liver S9 fractions (Table 3), the sulfation rates of the three MHFs showed no difference, or there was a small difference, between females and males. As a result, gender dependence exists in the sulfonation metabolism of MHFs.

SULTs are soluble in liver cytosol. Liver S9 fractions were prepared from fresh hepatocytes (both cytosol and microsomes). The cytosol fraction was composed of SULT, other metabolizing enzymes and other soluble proteins. Despite these differences, the present investigation, by using liver S9 fraction, will shed light on any possible sulfation of flavones in different genders and species.

Conclusions

Based on the results, the sulfation of the three MHFs was regiospecific, gender and species dependent. Multiple experiments using in-vivo perfusion model can be further used to determine the difference in species and gender for it not only supplies information on potential rates and metabolism routes but also provides guidelines for species dependence and gender dependence that can be used in preclinical studies.

Table 3 Gender-dependent sulfation rates of three flavones (7-MHF, 6-MHF and 4′-MHF) in liver S9 fractions prepared from the same animal but different genders

Sulfation rates (nmol/min/mg, mean ± SD)		Mouse		Rat		Dog	
		Female	Male	Female	Male	Female	Male
7-MHF	5 μM	0.0202 ± 0.0006	0.0228 ± 0.0003	0.0855 ± 0.0015*	0.1354 ± 0.0018	0.1557 ± 0.0023	0.1560 ± 0.0061
	10 μM	0.0250 ± 0.0008*	0.0220 ± 0.0008	0.0792 ± 0.0013*	0.1254 ± 0.0032	0.1685 ± 0.0067	0.1678 ± 0.0062
	40 μM	0.0277 ± 0.0007*	0.0213 ± 0.0006	0.1030 ± 0.0022*	0.1956 ± 0.0122	0.1153 ± 0.0174*	0.1529 ± 0.0041
6-MHF	5 μM	0.0009 ± 0.0001*	0.0005 ± 0.0001	0.0028 ± 0.0002*	0.0048 ± 0.0004	0.0582 ± 0.0003	0.0576 ± 0.004
	10 μM	0.0012 ± 0.0001*	0.0004 ± 0.0001	0.0041 ± 0.0002*	0.0066 ± 0.0001	0.0703 ± 0.0049	0.0734 ± 0.0003
	40 μM	0.0016 ± 0.0002*	0.0007 ± 0.0001	0.0061 ± 0.0004*	0.0099 ± 0.0002	0.1365 ± 0.0091*	0.1150 ± 0.0112
4′-MHF	5 μM	0.0564 ± 0.0098*	0.0402 ± 0.0019*	0.0130 ± 0.0010*	0.0329 ± 0.0065	0.0894 ± 0.0029	0.0913 ± 0.0021
	10 μM	0.0453 ± 0.0012*	0.0322 ± 0.0032	0.01780 ± 0.0008*	0.0429 ± 0.0013	0.0958 ± 0.0028	0.1001 ± 0.0048
	40 μM	0.0475 ± 0.0021*	0.0231 ± 0.0003	0.0148 ± 0.0025*	0.0327 ± 0.0035	0.1944 ± 0.0211	0.1873 ± 0.0017

Three different concentrations (5 μM, 10 μM, 40 μM) were used in the experiment ($n = 3$). Sulfation rates were calculated as nmol/min/mg protein. *$P < 0.05$ (Kruskall–Wallis test).

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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