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Comment on Uridine Diphosphate Glucuronosyltransferase Isoform-Dependent Regiospecificity of Glucuronidation of Flavonoids: Applicability of UV Spectrum Shifts in Identification of Glucuronidation Position in Flavones and Flavonols

In a paper published in this *Journal*, Singh et al.[1] investigated the regiospecific glucuronidation on the C3-, C5-, C7-, or C4′-hydroxy (OH) group of flavones and flavonols by a panel of uridine diphosphate glucuronosyltransferase (UGT) isoforms. The position of mono-*O*-glucuronide was determined on the basis of a UV-shift method developed by Singh and colleagues, which was recently published in this *Journal*.[2] They found that for flavones and flavonols, glucuronidation on C3-OH would cause a 13−30 nm hypsochromic shift in band I λ_{max} of 3-*O*-glucuronide (3-*O*-G) in relation to its corresponding aglycone. Glucuronidation on C5-OH and C4′-OH would cause a 4−10 nm hypsochromic shift in λ_{max} of bands II and I, respectively, whereas glucuronidation on C7-OH did not cause any shift in band I or II in all of the tested flavonoids (except for the 7-*O*-G of 3,7-dihydroxyflavone (3,7DHF)). The same research group adopted such a UV-based method in their recent UGT glucuronidation analyses.[3,4]

Detailed study on isoform-dependent regiospecific glucuronidation would help the elucidation of the structure−metabolism relationships of flavonoid glucuronidation by UGTs.[5] Because accurate identification of glucuronides is the prerequisite of regiospecificity research, it is essential to examine the reliability and robustness of Singh's UV-shift method.[2] Also, it deserves to be brought to the attention of the wider scientific community working on flavonoid analyses, metabolism, and pharmacokinetics.

First, we examined the UV spectra collected in a classic book titled *The Systematic Identification of Flavonoids*,[6] which is one of the earliest and yet most comprehensive collections of UV spectra of both flavonoids and their corresponding glycosides. The λ_{max} shifts induced by mono-*O*-glycosylation on flavones and flavonols prepared in methanol are summarized in Table 1. Notably, glycosylation at C7-OH mainly induced a hypsochromic shift in band I and a slight bathochromic shift in band II, which were in contrast to the findings of Singh[2] that no shifts were observed for 7-*O*-G. Glycosylation at C3-OH induced a prominent hypsochromic shift in band I with an insignificant shift in band II, which were consistent with Singh's findings.[2] We agree with Singh[2] that shifts in λ_{max} value might not be consistent across different conjugations. For example, for apigenin (5,7,4′HF) and luteolin (5,7,3′,4′HF), the λ_{max} (both bands I and II) of 7-*O*-glucoside and 7-*O*-rhamnoglucoside were virtually identical.[6] However, for quercetin (3,5,7,3′,4′HF), the band I λ_{max} of 3-*O*-rhamnoside (350 nm) and 3-*O*-galactoside (362 nm) differed prominently as shown in Table 1.

In addition to the above verification, we examined the applicability of this UV-shift method to the identification of flavonoid glucuronide in experimental settings. We previously described the in vitro enzyme kinetics for the glucuronidation of a series of monohydroxyflavones (MonoHFs)[7] and dihydroxyflavones (DHFs)[8] by human jejunum S9 and human jejunum microsome, respectively, using HPLC-UV for all of the sample analysis. For each studied DHF, two mono-*O*-glucuronides were found (confirmed by LC-MS/MS), but their identities were unknown because there were two OHs available for glucuronidation on each DHF (one OH at C7 and the other OH at another position). Therefore, we tried to identify the glucuronidation position of each mono-*O*-glucuronide by analyzing the UV spectra of both the aglycone and the two metabolites according to Singh's UV-shift method,[2] and the results are summarized in Table 2.

3,7DHF, 5,7DHF, and 7,4′DHF were tested by both Singh's group[2] and our group.[8] The UV shifts observed by the two groups were in good agreement, although, again, shifts in band I and/or II were found in our proposed 7-*O*-Gs. Singh also tested 6,7DHF,[2] but neither M1 (major metabolite) nor M2 (minor metabolite) of our 6,7DHF had UV shifts which resembled that of 6-*O*-G from Singh. The remaining three DHFs (7,8DHF, 7,2′DHF, and 7,3′DHF) were not tested by Singh, and therefore we need other inputs to identify the glucuronides. First, UV shifts observed in 2′-*O*-G and 3′-*O*-G (in relation to 2′HF and 3′HF, respectively) from our MonoHFs study[7] provided insights on the band shifts induced by glucuronidation on the B-ring OH groups. Moreover, the relative glucuronidation rates of these MonoHFs provided valuable information on the regiospecificity of human jejunum tissue toward different OH groups, which helped to differentiate the major metabolite (M1) from the minor metabolite (M2) in DHFs. Besides the shifts in λ_{max}, more attention should be paid to the changes in the relative heights of band I and band II peaks as they might provide additional information on glucuronidation position as demonstrated by 6,7DHF and 7,8DHF as shown in Table 2, although the significance of peak height in glucuronide identification requires further investigation. We agreed that a small wavelength shift (≤3 nm) might not be diagnostic.

The experimental conditions and detection methods applied in these three studies are summarized in Table 3. Technical specialists from the HPLC-UV (used by our group) and UPLC-UV (used by Singh's group) manufacture suggested that the wavelength resolutions of both Waters photodiode array detectors (PDAs) are 1.2 nm. Therefore, the detection method should not be a main source of discrepancy if Singh's group was employing the PDA from Waters. Besides the factors listed in Table 3, we acknowledge that the UV spectra and band shifts could be affected by other factors including concentrations of flavonoids/their glucuronides. Nevertheless, the aim of this Comment is to examine the applicability and reproducibility of

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Table 1. Summary of Effect of Mono-*O*-glycosylation on the λ_{max} of Bands I and II in the UV Spectra of Hydroxyflavones (HFs) Based on Spectra from Mabry et al.[6] [a,b]

HF aglycones and mono-*O*-glycosides	λ_{max} (nm) band II	λ_{max} (nm) band I	shift (nm) in bands I and II in relation to aglycones	comment on UV band shifts	consistent with Singh?[c]
5,6,7HF	274	323			
7-*O*-G	278	315	−8(I)/+4(II)	glucuronidation on C7-OH shifts both bands I and II	N
5,7,8HF	281	ND[d]			
7-*O*-G	274	ND	0(I)/−7(II)	glucuronidation on C7-OH hypsochromically shifts band II; the peak of band II remains much higher than that of band I in both aglycone and 7-*O*-G	N
5,7,4′HF	267	336			
7-*O*-Glu	268	333	−3(I)/+1(II)	glucosylation on C7-OH slightly shifts both bands I and II	Y/N
5,7H4′MF	269	327			
7-*O*-Glu	268	324	−3(I)/−1(II)	glucosylation on C7-OH slightly shifts both bands I and II hypsochromically	Y/N
5,7, 3′,4′HF	253, 267	349			
7-*O*-Glu	255, 267sh	348	−1(I)/+2(II)	glucosylation on C7-OH slightly shifts both bands I and II	Y/N
3,7, 3′,4′HF	248, 262sh	362			
3-*O*-Glu	254sh	340	−22(I)/?(II)	glucosylation on C3-OH blurs and/or shifts band II besides hypsochromically shifts band I	Y/N
3,5,7,3′,4′HF	255	370			
3-*O*-Rha	256	350	−20(I)/+1(II)	rhamnosylation on C3-OH hypsochromically shifts band I	Y
7-*O*-Rha	256	372	+2(I)/+1(II)	rhamnosylation on C7-OH slightly shifts both band I and II bathochromically	Y/N
3-*O*-Gal	257	362	−8(I)/+2(II)	galactosylation on C3-OH hypsochromically shifts band I, but the magnitude of shift is less than the suggested 13−30 nm[2]	Y/N
3,5,7,4′H3′MF	253	370			
3-*O*-Gal	255	357	−13(I)/+2(II)	galactosylation on C3-OH hypsochromically shifts band I	Y
3,5,7,8,3′,4′HF	261, 276	385			
7-*O*-Glu	261, 279sh	385	0(I)/0(II)	glucosylation on C7-OH does not shift band I or band II.; the peak of band II remains higher than that of band I in both aglycone and 7-*O*-Glu	Y
8-*O*-Glu	260, 273sh	380	−5(I)/−1(II)	glucosylation on C8-OH hypsochromically shifts bands I and II; the peak heights of bands II and I become comparable in 8-*O*-Glu	?
3,5,7,3′,4′H6MF	258	371			
3-*O*-Glu	261	355	−16(I)/+3(II)	glucosylation on C3-OH hypsochromically shifts band I	Y
7-*O*-Glu	259	373	+2(I)/+1(II)	glucosylation on C7-OH slightly shifts both bands I and II bathochromically	Y/N

[a]The UV spectra were prepared in spectroscopic grade methanol. [b]Abbreviations: G, glucuronide; Gal, galatoside; Glu, glucoside; HF, hydroxyflavone; MF, methoxyflavone; OH, hydroxyl; Rha, rhamnoside; sh, shoulder. [c]Symbols: Y, UV spectra and band shifts in good agreement with Singh and colleagues;[1,2] Y/N, partial agreement with small deviations; N , significant deviations; ?, uncertain due to insufficient information. [d]ND, no significant absorption peak detected.

Table 2. Identification of Position of Glucuronidation in Mono-*O*-glucuronides Generated from Dihydroxyflavones (DHFs) in Wong et al.[8] [a,b]

DHF aglycone and glucuronides formed	λ_{max} (nm) band II	λ_{max} (nm) band I	diagnostic shift (nm) in bands I and II in relation to aglycones	probable identification	deduction from UV spectra and/or rate of glucuronide formation	consistent with Singh?[c]
3,7DHF	252.2	340.0				
M1[d]	251.0	341.2	+1.2(I)/−1.2(II)	7-*O*-G	UV: 13−30 nm hypsochromic shift in band I of M2 indicated 3-*O*-G; Singh[2] found 4.7 nm hypsochromic shift in band II and no shift in band I in 7-*O*-G of 3,7DHF	Y/N
M2[d]	251.0	313.8	−26.2(I)/−1.2(II)	3-*O*-G		Y
5,7DHF	267.5	312.6				
M1	267.5	305.4	−7.2(I)/0(II)	7-*O*-G	UV: 4−10 nm hypsochromic shift in band II of M2 indicated 5-*O*-G; however, no shift in band I of M1 should be observed if M1 was 7-*O*-G	N
M2	260.4	317.3	+4.7(I)/−7.1(II)	5-*O*-G	rate: C5-OH was not the favored position in chrysin, and M2 was found in trace amount	Y
6,7DHF	265.2	313.8				
M1	259.2	313.8	0(I)/−6(II)	6-*O*-G?	UV: Singh[2] found 9.6 nm hypsochromic shift in band I and no shift in band II in 6-*O*-G of 6,7DHF, with no change in band I or II in 7-*O*-G; Singh[2] showed that the peak heights of band II remained much higher than those of band I in both 6,7DHF and 7-*O*-G, whereas in 7-*O*-G the peak heights of bands II and I became comparable; in our study bands II and I had comparable heights in 6,7DHF and M2, whereas in M1 the band I peak became higher than the band II peak	?
M2	265.2	312.6	−1.2(I)/0(II)	7-*O*-G?	rate: CL_{int} of 6HF in human jejunum S9 was 10 times higher than that of 7HF[7]	?
7,8DHF	265.2	ND[e]				
M1	256.9	311.4	0(I)/−8.3(II)	8-*O*-G?	UV: similar to the mono-*O*-glycosylation of 3,5,7,8,3′,4′HF and 5,7,8HF (Table 1), the peak of band II remained higher than that of band I in both aglycone and M2 (7-*O*-G ?), whereas the peak heights of bands II and I became comparable in M1 (8-*O*-G?); together with the observations in 6,7DHF, glucuronidation on C6-OH or C8-OH of flavones with 6,7-*o*-diOH or 7,8- *o*-diOH seems to boost the band I peak height and/or lower the band II peak, whereas glucuronidation on C7-OH has no apparent effects on the relative heights of bands I and II	?
M2	264.0	ND	0(I)/−1.2(II)	7-*O*-G?		?
7,2′DHF	ND	309.0				
M1	ND	312.6	0(I)/+3.6(II)	2′-*O*-G?	UV: according to Zhang,[7] glucuronidation of 2′HF caused a bathochromic shift in band I (1.2 nm), whereas glucuronidation of 7HF caused a hypsochromic shift in band I (1.2 nm)	?
M2	252.2	304.3	0(I)/−4.7(II)	7-*O*-G?		?
7,3′DHF	234.5	309.0				
M1	254.5	311.4	+2.4(I)/+20(II)	3′-*O*-G	UV: according to Zhang,[7] glucuronidation of 3′HF caused bathochromic shifts in band II (12.9 nm) and band I (1.2 nm)	?
M2	225.1	309.0	−9.4(I)/0(II)	7-*O*-G	rate: CL_{int} of 3′HF in human jejunum S9 was the highest among the studied MonoHFs and was 12 times higher than that of 7HF,[7] whereas only a trace amount of M2 was detected	?
7,4′DHF	ND	330.4				
M1	253.3	324.5	0(I)/−5.9(II)	4′-*O*-G	UV: Singh[2] found 9.5 nm hypsochromic shift in band I and no shift in band II in 4′-*O*-G of 7,4′DHF, with no change in band I or II in 7-*O*-G	Y
M2	253.3	329.2	0(I)/−1.2(II)	7-*O*-G	rate: CL_{int} of 4′HF in human jejunum S9 was 3 times higher than that of 7HF[7]	Y/N
7HF	251.0	309.0				
M1	252.2	307.8	−1.2(I)/+1.2(II)	7-*O*-G	UV: no shift in band I or II of M1 should be observed if M1 was 7-*O*-G	Y/N

[a]The UV spectra were obtained by HPLC-UV analysis of in vitro incubation sample with an original concentration of DHF at 63 μM. [b]Abbreviations: CL_{int}, intrinsic clearance; DHF, dihydroxyflavone; G, glucuronide; HF, hydroxyflavone; OH, hydroxyl. [c]Symbols: Y, UV spectra and band shifts in good agreement with Singh and colleagues;[1,2] Y/N, partial agreement with small deviations; N, significant deviations; ?, uncertain due to insufficient information. [d]M1, major metabolite (with larger peak area in HPLC-UV chromatogram); M2, minor metabolite (with smaller peak area in HPLC-UV chromatogram). [e]ND, no significant absorption peak detected.

Table 3. Comparison of Experimental Settings Used in the Three Studies

	Singh et al. (2011)	Wong et al. (2009)	Mabry et al. (1970)
analytical apparatus	Waters Acquity UPLC with photodiode array detector	Waters 2695 HPLC with Waters 996 photodiode array detector	Beckman DB-G spectrophotometer
photodiode array detector wavelength resolution	1.2 nm	1.2 nm	N/A
mobile phases	acetonitrile; ammonium acetate buffer (pH 7.5)	acetonitrile; methanol; 20 mM sodium phosphate buffer (pH 4.6)	N/A
origin/supplier of flavonoid standards	all from Indofine Chemical	all from Indofine Chemical (except chrysin from Aldrich Chemical)	unknown
preparation of sample	glucuronides in in vitro reaction system extracted by solid phase extraction and reconstituted in 30% acetonitrile in water	reaction system from in vitro glucuronidation experiment centrifuged at 16000g and supernatant injected to HPLC	flavonoids dissolved in spectroscopic grade methanol (paper chromatography might be employed to purify the flavonoids)

Singh's method in other laboratories under similar experimental conditions.

In conclusion, Singh's method[2] can be used with confidence to identify the glucuronidation on C3-OH based on the prominent hypsochromic shift in band I. However, for glucuronidation that occurs at C5-, C6-, C7-, or C4′−OH, several inconsistencies between Singh's results[2] and those from others were observed in terms of the band involved (band I or band II), the nature of the shifts (hypsochromic, bathochromic, or no shift), and magnitude of the shifts. For the remaining positions (C8-, C2′-, or C3′-OH), information on the band shift after glucuronidation is still lacking. Further studies are needed to verify the reliability of the proposed UV-shift method in the identification of glucuronidation position in flavonoids before the application of such a method can be generalized to different regiospecificity studies and to other laboratories.

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Notes

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