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Response to Comment on Uridine Diphosphate Glucuronosyltransferase Isoform-Dependent Regiospecificity of Glucuronidation of Flavonoids

We are responding to comments from Dr. Yin Cheong Wong and Dr. Zhong Zuo of the Chinese University of Hong Kong on our recently published paper.[1] It is noted that their comments essentially were made on our earlier paper.[2] Before responding to these comments, we would like to restate the objective of our work.[2] The main objective was to develop an easy and economical UPLC-DAD method for determining the position(s) of mono-*O*-glucuronidation on the structure of flavones and flavonols on the basis of the diagnostic spectral shift in λ_{max} of band I and/or band II. To achieve this objective, 36 compounds (mono-, di-, tri-, and tetrahydroxyflavones) were used, which generated one or more mono-*O*-glucuronide using different UGT isoforms. On the basis of the diagnostic shifts in various monohydroxyflavones, the method was validated using di-, tri-, and tetrahydroxyflavones. The robustness of the method was also established using different UPLC methods, mobile phases, and more flavonols in our later publications.[3−5]

Drs. Wong and Zuo in their comments showed that according to the Mabry book[6] both band I and band II λ_{max} of a couple of compounds changed by >4 nm with the glucuronidation of the 7-*O* position, which is inconsistent with our results. We point out that the specific solvent used during spectral analysis by Mabry et al. was sodium acetate,[6] which is different from ours and could be responsible for the difference in shift, in addition to the fact that different types of substitutions could cause different spectral shifts. We have tested 11 compounds with 7-*O*-glucuronidation to validate our method. We believe that if the UPLC method, mobile phase, and detector specification are kept consistent with our conditions, similar results could be obtained in other laboratories. However, even if the method and instrument specifications are changed, other laboratories can easily develop and validate their own diagnostic shifts using a series of diagnostic compounds, as shown in our later publications.[3−5] Therefore, this conclusion was based on a published value from a book, which could be a typographical errors. Proper and complete experimental investigation of these two compounds would be needed to determine if the published values are correct.

Drs. Wong and Zuo showed that in 7-HF, 7,4′DHF, 3,7DHF, 6,7DHF, 7,8DHF 7-*O*-glucuronide substitution caused a spectral shift of ±1.2 nm in the λ_{max} of bands I and II, whereas our data showed no change. We have used the slit width of 2.4 nm such that spectral resolution is relatively low but sensitivity is high, as we usually use during our quantitative analysis (note that Wong and Zuo used a high-resolution setting, in addition to different mobile phase and gradient in their studies). If we had decreased the slit width, we would have gained specificity or more accurate wavelength, but would have been limited for identification of position in samples with low metabolite concentrations. Keeping the slit width to ±2.4 nm and sacrificing resolution have helped us to analyze experimental samples with lower metabolite concentrations for all our studies in later publications.[3−5] Therefore, we did not consider a wavelength change of ≤4 nm to be significant in the case of any spectrum as it could very well be caused by the instrument variability rather than a real spectral change.[2] The only significant difference with our data was seen in the spectral shift of 7-*O*-glucuronide of 5,7DHF. However, we have not seen any change in wavelength of 7-*O*-glucuronide of 5,7DHF in samples from different sources (Caco-2, S9 fraction, liver microsomes, etc.) or using different UPLC methods and mobile phases (unpublished data). We have no explanation for this apparent difference.

We did not discuss glucuronidation at positions 2′, 3′, and 8 in our paper[2] as there were not enough compounds with 2′-, 3′-, 8-hydroxyl groups available to perform a validation for diagnostic wavelength shift. Also, we found that the rate of glucuronidation of monohydroxyflavones by recombinant UGTs or liver/jejunum S9 fractions alone cannot be used to determine the position of glucuronidation in di- and trihydroxyflavones, as our studies have shown that regiospecificity and selectivity of a hydroxyl group might change drastically upon substitution of one or more hydroxyl groups at different positions in a monohydroxyflavone.[4] For example, a 4′-*O*-hydroxyl group is glucuronidated much more quickly in 5,4′DHF, whereas not so quickly in 7,4′DHF, by UGT1A1.[1] Therefore, the exact positions of substitution of monoglucuronides from compounds 7,2′DHF and 7,3′DHF remain uncertain.

Finally, Drs. Wong and Zuo discussed and compared changes in peak heights of bands I and II in deducing glucuronide position of metabolites of 6,7DHF. However, we never discussed and compared changes in peak heights of different glucuronides formed in our paper. Studies in our laboratory have demonstrated that peak heights of bands I and II were highly dependent on the concentration of metabolite in a sample and subject to change as the rate of glucuronidation changes with each UGT isoform. Hence, in our opinion, changes in peak height were not validated as a useful method of deducing the position of glucuronidation.

However, we agree with Drs. Wong and Zuo regarding the limitations of our method in the cases of identification of the position of glucuronidation in certain compounds, for example, between 6-*O* and 7-*O* positions in 6,7DHF and 7-*O* and 3′-*O* positions in quercetin as discussed in our paper.[1] Therefore, in our recent publication, we have proposed the successful use of elution order of various glucuronides and of strict regioselectivity of UGT isoforms as a complementary method in the cases when the identification of glucuronidation position cannot be deduced by UV spectral shift method alone.[7]

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Notes
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