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Letter to the Editor

Dear Editor

Interference of a sulfate conjugate in quantitative liquid chromatography/tandem mass spectrometry through in-source dissociation

Over the last 15–20 years, high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) has played an increasingly important role in the quantification of analytes in complex biological matrices, due to its superior sensitivity and specificity. The separation of analytes in LC/MS/MS is achieved first by their physiochemical properties by the chromatographic separation and secondly by the mass-to-charge resolution provided by the mass spectrometer. Due to this specificity of the mass spectrometer, multiple compounds can be quantified simultaneously within a very short analytical run time. This property of LC/MS/MS has been appreciated especially by the pharmaceutical industry: chromatographic run times of less than 3 min are commonly applied to allow the high throughput of samples.[1,2] MS/MS provides an accurate specific method for most analytes by coupling a specific precursor ion with a unique fragment ion. There are cases however where interferences can arise. In-source dissociation of metabolites can potentially produce ions identical to the precursor ions of the compound under study.[1–3] The most commonly reported interferences that can undergo in-source dissociation are different types of glucuronide conjugates, including acyl glucuronides, carbamoyl glucuronides and *N*- and *O*-glucuronides.[3–5] In addition to glucuronides, in-source dissociation of *N*-oxide metabolites has also been documented.[6] In this communication, we report the interference arising from a sulfate metabolite in LC/ESI-MS/MS. To the best of our knowledge, there have been no reports on interference generated from a sulfate conjugate. The sulfate conjugate was almost undetected in our investigation because it was eluted almost at the same time as the parent drug under the chromatographic conditions initially used. The parent compound we investigated here is biochanin A, one of the most commonly consumed isoflavones.[7] The samples were collected in transport buffer (HBSS) when we evaluated the transport of biochanin A across MDCK (Madin-Darby canine kidney) cell monolayers.

LC/MS/MS was performed using an Applied Biosystems API 3000 triple-quadruple tandem mass spectrometer linked to a TurboIonspray interface and a Shimadzu Prominence liquid chromatograph. To minimize the ion suppression caused by the high concentration of salts in the transport buffer (HBSS), the flow from the LC column was diverted to waste for the first 2 min using a diversion valve to prevent the early eluting salts from entering the LC/MS interface. Conditions for MS analysis of biochanin A included an ion spray voltage of −4500 V, and a temperature of 350°C. Nebulizer and curtain gas flow were 10 mL/min and 8 mL/min, respectively. The fragment was induced with a collision energy of −30 eV. The optimized declustering potential (DP), focusing potential and collision cell exit potential were −60, −175 and −30 V, respectively. The MS was performed in a negative ion mode in multiple reaction monitoring (MRM) mode. The *m/z* ratios of precursor ion and product ion of biochanin A were 283 and 268, respectively. A XTerra MS C18 column (2.1 × 150mm i.d., 3.5 μm; Waters Corporation, Milford, MA, USA) was used with a flow rate of 200 μL/min. The mobile phase we used initially consisted of 60% acetonitrile with 0.1% formic acid (mobile phase B) and 40% of water with 0.1% formic acid (mobile phase A). A peak with a clear shoulder was observed in the channel for biochanin A (*m/z* 283→268), as shown in Fig. 1(A). As discussed by Liu *et al.*,[4] the use of a deteriorated column or an inappropriate reconstitution solvent is usually the common reason contributing to a peak shoulder. However, neither of the reasons was applicable to our case since we used a brand new column and appropriate reconstitution solution. After the composition ratio of mobile phases A and B was adjusted to 50:50, the shoulder was resolved into a second peak, as shown in Fig. 1(B). Since glucuronide conjugates are the most commonly reported interferences and glucuronidation has been reported to be one of the major pathways regarding the metabolism of biochanin A, we hypothesized that this interference peak was a biochanin A glucuronide conjugate. However, no peak was observed when we added the precursor to product ion transition for biochanin A glucuronide (459→283) to our MS condition. Therefore, this interference peak either is a non-glucuronide metabolite or is a glucuronide conjugate undergoing complete in-source dissociation under our current MS conditions. To further investigate whether this interference was a biochanin A glucuronide conjugate, an aliquot (50 μL) of sample was incubated with an equal volume of buffer containing no enzyme or β-glucuronidase (in 350 mM phosphate buffer, pH 6.8). No change of either peak was observed when the sample was incubated with 50 U glucuronidase, indicating that this interference peak is not a glucuronide conjugate (data not shown). For those compounds with phenolic group(s), in addition to O-glucuronidation, O-sulfation is also a common metabolic pathway. Therefore, we speculated that the interference peak observed might be a sulfate metabolite. Consistent with our hypothesis, enzyme hydrolysis of transport samples using 5 U sulfatase resulted in the disappearance of the interference peak and an increase in the biochanin A aglycone peak, indicating that the interfering peak was a sulfate metabolite. In addition, we added the precursor to product ion transition for biochanin A sulfate (363→283) to our MS method and a peak was observed at the same retention time as our interference peak observed in the 283→268 channel (shown in Fig. 2). The fragment at *m/z* 283 was strong evidence that this peak contained biochanin A. The precursor ion of 363 indicated that this metabolite contained a monosulfate moiety

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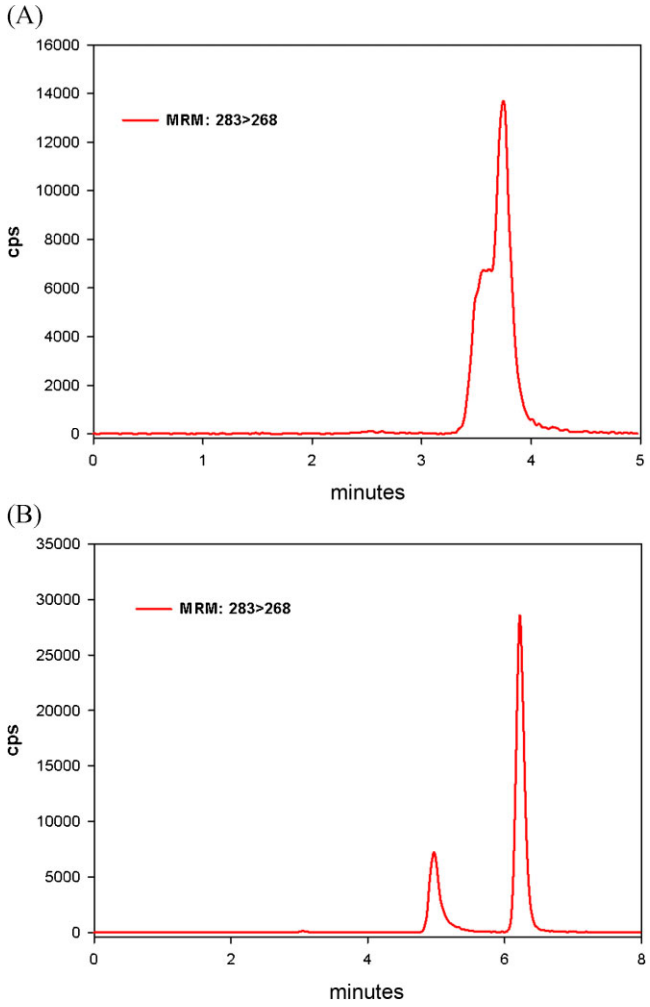

Figure 1. MRM chromatograms (negative ion mode) of biochanin A sample when the mobile phase consisted of (A) 60% of mobile phase B (acetonitrile with 0.1% formic acid) and 40% of mobile phase A (water with 0.1% formic acid); or (B) 50% of mobile phase B and 50% of mobile phase A. Biochanin A was detected under the channel of 283/268.

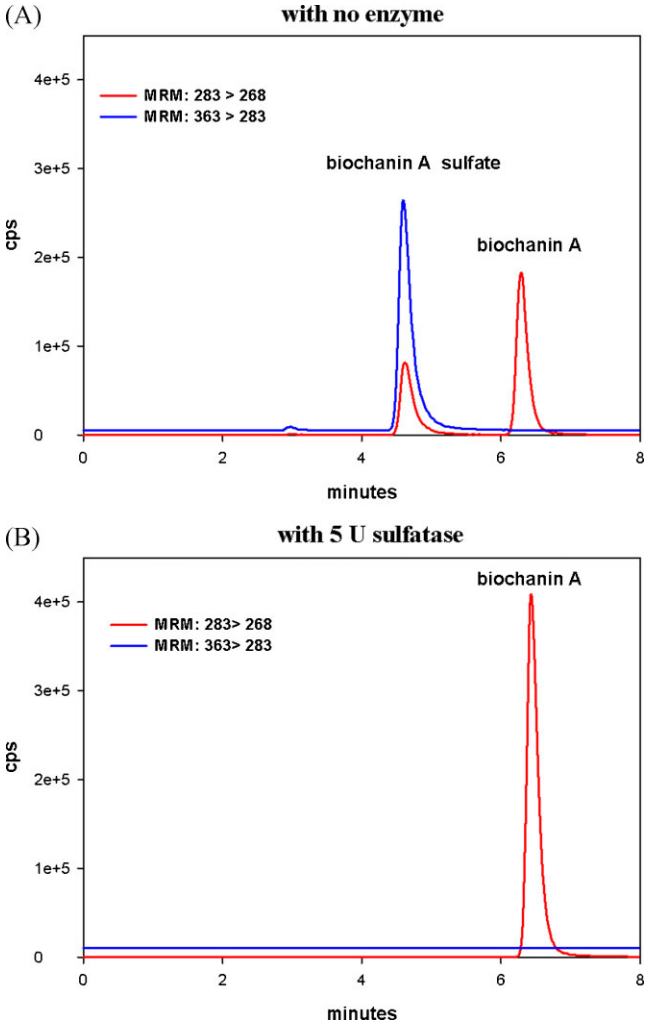

Figure 2. MRM chromatograms (negative ion mode) of an aliquot of a 50 μL sample (from transport study) incubated with an equal volume of buffer containing (A) no enzymes; (B) 5 U of sulfatase. The red line represents the chromatogram obtained under MRM of 283/268 (for the measurement of biochanin A). The blue line represents the chromatogram obtained under MRM of 363/283 (for the measurement of biochanin A sulfate). Biochanin A was eluted at about 6.3 min and biochanin A sulfate was eluted at about 4.7 min.

(molecular weight (mw) 80). The proposed biochanin A sulfate conjugate formation (in MDCK cells) and in-source dissociation during the ionization process (in MS) are shown in Fig. 3.

There are reports in the literature of different MS parameters as the cause of the in-source dissociation. For example, Yan *et al.*[5] reported that source temperature had little effect on the in-source glucuronide dissociation and the presence of glucuronide interference was dependent on the cone voltage. However, the data from another research group[4] clearly showed temperature-dependent in-source dissociation: the carbamoyl glucuronide conjugate displayed slight in-source fragmentation at 300°C and much more fragmentation was obtained when the temperature was increased to 400°C. To investigate whether in-source dissociation of the sulfate conjugate was also sensitive to certain MS parameter(s) in our current study, we further examined the stability of biochanin A sulfate at different MS source parameters using two MS instruments from different manufacturers. Instrument 1 was a Thermal Scientific TSQ quantum mass spectrometer and instrument 2 was an Applied Biosystems API 3000 triple-quadruple tandem mass spectrometer. The electrospray ionization (ESI) method was used in both instruments. As shown in Fig. 4, the in-source dissociation of the sulfate metabolite is dependent on the temperature in instrument 1. A higher degree of in-source fragmentation was observed as capillary temperature was increased. When samples were analyzed using instrument 2, the in-source dissociation of the sulfate conjugate demonstrated both temperature- and DP-independence (data not shown). Our results indicated that the stability of the sulfate conjugate may be dependent on not only certain MS parameters, but also different instruments. It should be noted that the ionization process used in the current study is ESI, a process usually operated at a relatively low temperature. Since atmospheric pressure chemical ionization (APCI) is also a widely used ionization method and usually requires higher temperatures than ESI, extra caution should

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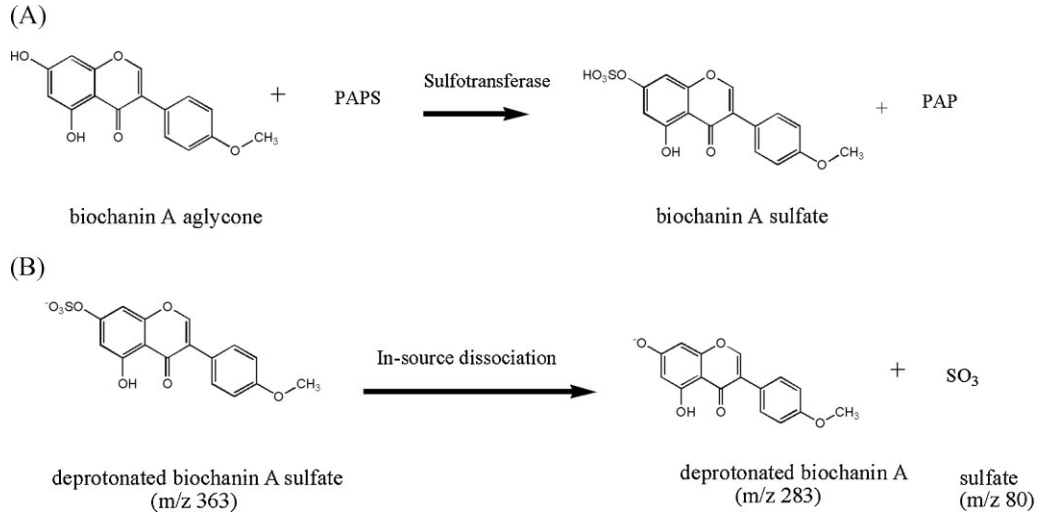

Figure 3. Proposed biochanin A sulfate formation (in MDCK cells) and in-source dissociation in the ionization process (in MS). In-source dissociation of sulfate from biochanin sulfate in ESI generates a fragment ion which is identical to the precursor ion of biochanin A.

be exercised when APCI is used to analyze samples containing both target compound and sulfate conjugates.

Sulfotransferase-catalyzed conjugation is one of the most important biotransformation reactions and various endobiotics and xenobiotics are substrates of sulfotransferases (SULTs).[8,9] It has been reported that many of the substrates metabolized by UDP-glucuronosyltransferases (UGTs) are also substrates for SULTs.[10,11] Previously, glucuronide metabolites, but not sulfate metabolites, were reported to interfere with the parent compound thorough in-source dissociation. One possible reason is that in many of the previous studies, samples were collected after incubation of a drug in microsomal preparations, a widely used *in vitro* system that does not contain SULTs. Our samples were collected from transport buffer when the transport of biochanin A across MDCK cell monolayers was investigated.

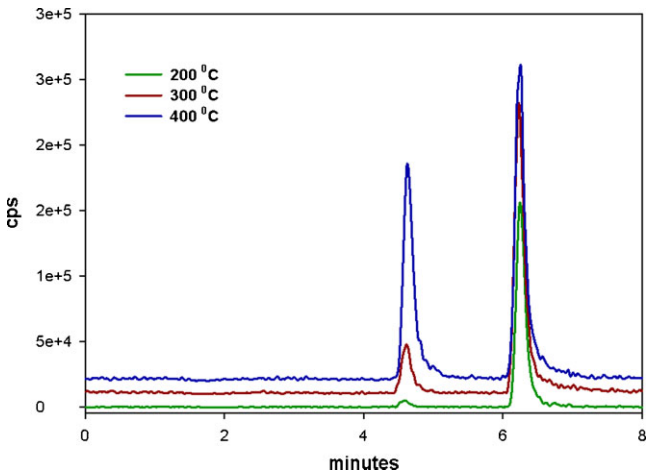

Figure 4. MRM chromatograms (negative ion mode) of a sample containing biochanin A and its sulfate metabolite in ESI mode using the Thermal Scientific TSQ Quantum Ultra mass spectrometer. The sulfate metabolite displays (bottom) slight in-source dissociation at 200°C; (middle) moderate in-source dissociation at 300°C; and (top) extensive in-source dissociation at 400°C. The chromatograms were obtained under MRM of 283/268.

It should be noted that Caco-2 and MDCK cells, both of which have SULT expression, have been used extensively in studies to characterize drug metabolism and drug permeability.[12]

In conclusion, potential interferences from sulfate conjugates have been investigated and extra caution should be taken when establishing conditions for a LC/MS/MS assay to detect a new compound that can be metabolized mainly by UGT and/or SULT.

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