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Identification of isomeric flavonoid glucuronides in urine and plasma by metal complexation and LC-ESI-MS/MS

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Noncovalent complexes were used for structural determination and isomer differentiation of flavonoid glucuronides. Several flavonoid glucuronides including naringenin-7-*O*-glucuronide, synthesized here for the first time, were used as test compounds. Electrospray ionization quadrupole ion trap mass spectrometry with collision-induced dissociation (CID) was used to analyze complexes of the form [Co(II) (L-H) (Aux)]$^+$ and [Co(II) (L-H) (Aux)$_2$]$^+$, in which L is the flavonoid glucuronide and Aux is a phenanthroline-based ligand. These complexes yielded characteristic fragmentation patterns that facilitated assignment of the substitution position of the glucuronides. The methods were adapted to liquid chromatography/tandem mass spectrometry (LC-MS/MS) with postcolumn cobalt complexation and were tested on extracts from biological fluids. The metabolites naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide were detected in human urine following the consumption of grapefruit juice. Isomeric quercetin glucuronides were identified and differentiated after spiking rat plasma at the 1 μM level, proving that the new methods are effective at biologically relevant concentrations. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: flavonoid glucuronides; isomerism; metabolism; metal complexes; tandem mass spectrometry

INTRODUCTION

The investigation of metabolic pathways is a vital step in understanding the mechanism of action of bioactive molecules. In the case of flavonoids, recent work has begun to shed light on the biotransformation of these compounds.[1] It is now known that while flavonols, flavanones, and flavones are consumed in the diet mainly as glycoside conjugates, the saccharide portions are removed during absorption.[2–4] Because metabolism occurs rapidly, it is mainly the glucuronidated, sulfated, and methylated derivatives that are present in plasma following consumption.[5,6] As a result of these findings, concerns have been raised about the reliability of much of the data on the bioactivity of flavonoids.[7,8] Most studies have involved either flavonoid aglycons or glycosides that are not present for any appreciable amount of time in the body, and the doses applied are often at much higher levels than are achieved after eating foods rich in flavonoids. It has therefore been suggested that *in vitro* studies of flavonoids should use metabolites rather than commercially available aglycons and glycosidic forms found in foodstuffs.[7,8] However, the problem remains that far less is known about the *in vivo* metabolites than about their precursors, and relatively few analytical methods have been developed for studying the metabolites. The availability of flavonoid metabolite standards is extremely limited.

As with all flavonoid species, determining the substitution patterns of flavonoid glucuronides is a considerable challenge. Nuclear magnetic resonance (NMR) spectroscopy may be used to obtain this information,[9,10] but this technique is less applicable to dietary metabolites, which are present in very low concentrations. A UV-vis spectroscopic method[11] has also been used to determine the position of the glucuronide moieties on flavonoid metabolites,[12] but this is a complicated method involving the use of several shift reagents. Liquid chromatography/tandem mass spectrometry (LC-MS^n) is in many ways an ideal method for analyzing and identifying flavonoid metabolites owing to its high sensitivity, applicability to complex mixtures, and ability to provide structural information. However, full structural characterization is often not possible by mass spectrometry (MS). While flavonoid glucuronides can be identified by the characteristic loss of the glucuronide moiety (−176 Da) upon dissociation, it has not yet been possible to determine the location of the glucuronide by mass spectrometry.[13–17] Previous work has shown that the positions of saccharide moieties have a large effect on the bioactivity of flavonoids,[18,19] and a similar effect was noted for conjugated metabolites.[12] Thus, the ability to elucidate the structure of flavonoid metabolites as comprehensively as possible would be extremely useful.

Metal complexation strategies[20–25] have recently been used to determine specific structural features of flavonoid

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conjugates by tandem mass spectrometry. Many flavonoids are natural chelators and when mixed in solution with metal salts will often spontaneously form metal complexes.[26–30] These complexes typically provide more information upon collision-induced dissociation (CID) than uncomplexed flavonoid species such as deprotonated flavonoids. The metal complexation strategy has been used to determine common disaccharide configurations of flavonols, flavones, and flavanones, including the elucidation of the nature of the attached sugars (i.e. a rutinose sugar at the 3 or 7 position or an isomeric neohesperidose sugar at the 7 position).[20–23] Other metal complexes containing monoglycosyl flavonols, flavones, and flavanones have been used to determine the specific saccharide location among the five most commonly encountered glycosylation positions.[24,25] Even diastereomeric sugars, such as glucose and galactose, can be differentiated by metal complexation with tandem MS.[25] Some of these methods have been adapted to LC-MS analysis of food extracts, proving sufficient simplicity and sensitivity for the on-line analysis of complex mixtures.[22,25]

Here, we present metal complexation methods that allow the position of glucuronidation to be determined for some flavonoid metabolites containing a single glucuronide moiety. Four isomeric quercetin monoglucuronides can be differentiated on the basis of characteristic fragmentation patterns observed upon CID of the complexes. Moreover, consistent product ion signatures were found for complexes involving 7-*O*-glucuronides and 3-*O*-glucuronides in the flavonol, flavone, and flavanone groups. The metal complexation methods were adapted for the analysis of flavonoid

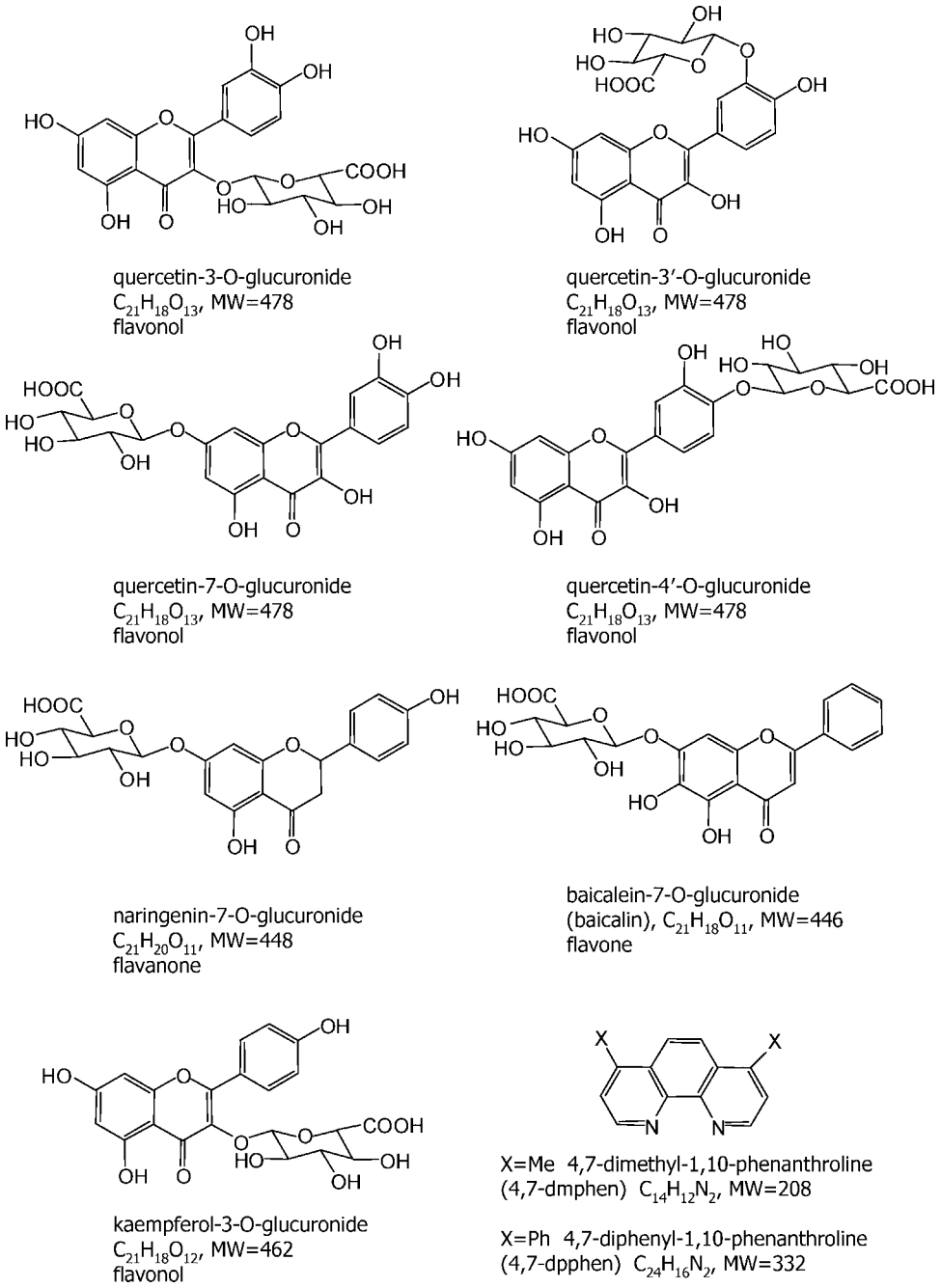

Figure 1. Structures and molecular weights of the compounds in this study.

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glucuronides in complex biological matrices via LC-MS with postcolumn complexation. In this way metabolites extracted from human urine and rat plasma were identified.

EXPERIMENTAL

Materials

Quercetin-3-*O*-glucuronide,[31] quercetin-3′-*O*-glucuronide,[31] quercetin-4′-*O*-glucuronide,[32] quercetin-7-*O*-glucuronide,[32] and naringenin-7-*O*-glucuronide were synthesized at the Institute of Food Research. The novel synthesis of naringenin-7-*O*-glucuronide from 4′-acetylnaringenin (in a manner analogous to the synthesis of daidzein-7-glucuronide[33]) is described in a later section. The above compounds were analyzed in their sodium salt form. Baicalin (baicalein-7-*O*-glucuronide) was purchased from Extrasynthèse (Genay, France). Kaempferol-3-*O*-glucuronide was purchased from Apin Chemicals (Abingdon, UK). Cobalt(II) bromide, 4,7-dimethyl-1,10-phenanthroline (4,7-dmphen), and 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen) were purchased from Aldrich (Milwaukee, WI). Structures of these molecules are depicted in Fig. 1. Rat plasma was purchased from Pel-Freez Biologicals (Rogers, AR). Solvents used were of the highest grade available, and were dried, where specified, over freshly activated 3 Å molecular sieves. All other commercial materials were used without further purification. ^{1}H and ^{13}C NMR spectra were run on a JEOL EX-270 spectrometer. The sample temperature was 21 °C and chemical shifts were referenced to residual solvent absorption.

Purification of synthetic intermediates and conjugates by liquid chromatography

Medium pressure liquid chromatography (MPLC)[34] used prepacked silica cartridges (ISOLUTE Flash Si, Argonaut Technologies) and ultraviolet (UV) detection. Analytical high-performance liquid chromatography (HPLC) used a 5 μm Lunar column (250 × 4.4 mm) eluted at 1 ml/min. The two HPLC solvents were 0.1% trifluoroacetic acid (A) and acetonitrile (B). The elution method was isocratic, using 20% B for 5 min followed by a 30-min gradient to 90% B. The eluate was monitored by UV detection at 205 and 280 nm. LC-MS analyses were performed on a Micromass Quattro II mass spectrometer (Manchester, UK) equipped with an electrospray ionization (ESI) source. Preparative HPLC of the synthetic conjugates used a 5 μm Prodigy ODS3 column (Phenomenex Inc., 250 × 21.2 mm + 60 × 21.2 mm guard) eluted at 5 ml/min. The preparative HPLC method used 20% B isocratic for 15 min, followed by a 75-min gradient to 72.5% B. The synthesis is depicted in Fig. 2 and described in detail below.

Preparation of 4′-*O*-acetylnaringenin, 2

4′,7-di-*O*-acetylnaringenin[35] **1** (3.0 g, 8.42 mmol) was dissolved, with stirring, in tetrahydrofuran (300 ml) at 20 °C. 1 M aqueous imidazolium hydrochloride (300 ml, pH 7.0) was added, and stirring was continued for 2.5 h. Acetic acid (40 ml) was added, and the mixture was extracted with CH_2Cl_2 (1 × 300 ml). The organic phase was washed with water (1 × 100 ml), dried ($MgSO_4$) and evaporated.

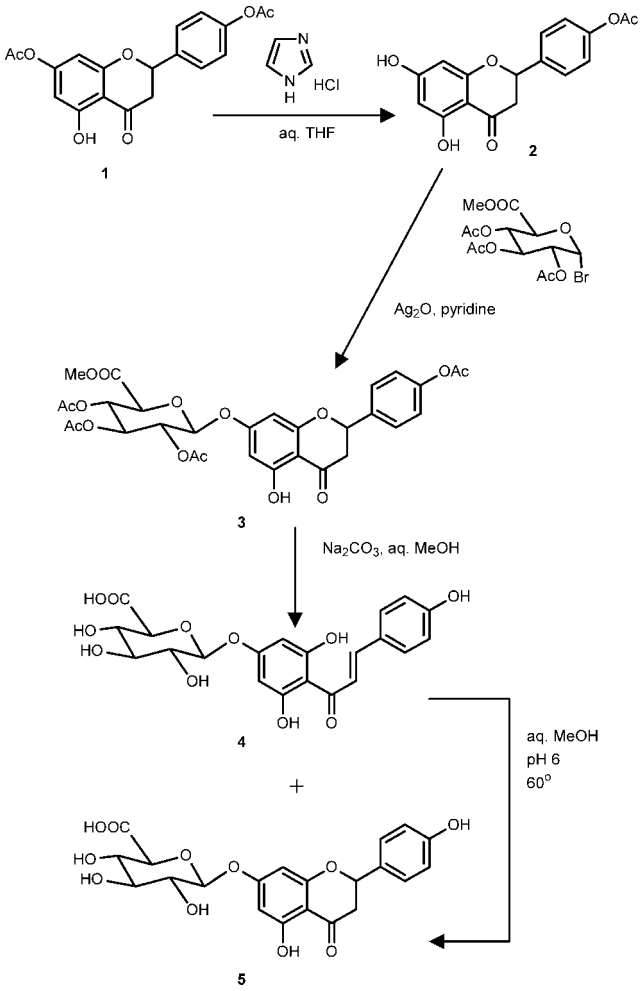

Figure 2. Synthesis of naringenin-7-*O*-β-D-glucuronide, **5**.

The residue was co-evaporated with toluene (2 × 20 ml) and dichloromethane (1 × 50 ml) and purified by MPLC (50 g silica ISOLUTE SI II cartridge, isocratic elution with 2% MeOH/98% CH_2Cl_2) to give 4′-*O*-acetylnaringenin **2** (1.63 g, 63%).

2. ^{1}H NMR ($CDCl_3$): δ 12.09 (s, 1 H, 5-OH), 7.46 (AAB′B′ system, 2H, $J_{3',2'}$ 8.2 Hz, H-3′, H-5′), 7.14 (AAB′B′ system, 2H, H-2′, H-6′), 5.99 (d, 1 H, $J_{8,6}$ 2.3 Hz, H-8 or H-6), 5.97 (d, 1 H, H-6 or H-8), 5.40 (dd, 1 H, $J_{2,3eq.}$ 3.0 Hz, $J_{2,3ax.}$ 12.9 Hz, H-2), 3.05 (dd, 1 H, $J_{3ax.,3eq.}$ 17.5 Hz, H-3-ax.), 2.80 (dd, 1 H, H-3eq.)

Preparation of naringenin-7-*O*-β-D-glucuronide, 5

Intermediate **2** (0.5 g, 1.59 mmol), powdered 3 Å molecular sieves (0.5 g), methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (0.95 g, 2.39 mmol, 1.5 equiv.), and dry pyridine (10 ml) were stirred, in the dark, under Ar for 5 min. Ag_2O (0.55 g, 1.5 equiv.) was added and stirring was continued. After 16 h, aqueous KCl (10%, 50 ml) and aqueous acetic acid (10%, 250 ml) were added, and the mixture was filtered through celite. The latter was washed with water (2 × 200 ml), and the crude product eluted with acetone (250 ml). The acetone extract was evaporated to give a light brown glass (0.80 g), which was purified by MPLC (20 g silica, 40% ethyl acetate/60% hexane, isocratic elution) to

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give the crude, protected, glucuronidated product **3**. The latter was suspended in aqueous methanol (50% v/v, 120 ml), aqueous Na_2CO_3 (0.5 M, 3.6 ml) was added, and the mixture was stirred under Ar for 2.5 h. The resultant solution was adjusted to pH 3.4 with Dowex 50W ion-exchange resin (H^+ form), filtered, and evaporated. The residue, which consisted mainly of a mixture of **5** and naringenin chalcone 7-glucuronide **4**, was dissolved in aqueous methanol (50% v/v, 20 ml), adjusted to pH 6.0 with aqueous NaOH (0.1 M), and heated, under Ar, at 60 °C for 66 h. The resultant crude **5** was virtually free of **4** and was purified by preparative HPLC. **5** was dissolved in aqueous methanol (50% v/v, 20 ml), adjusted to pH 6.8 with aqueous NaOH (0.1 M), and evaporated. The yield of **5** (sodium salt) was 49 mg (7% from **2**).

4. ^{1}H NMR (CD_3OD): δ 8.04 (d, 1 H, $J_{\alpha,\beta}$ 15.5 Hz, H-α), 7.73 (d, 1 H, H-β), 7.49 (AAB′B′ system, 2 H, $J_{3,2}$ 8.9 Hz, H-3, H-5), 6.82 (AAB′B′ system, 2 H, H-2, H-6), 6.10 (s, 2H, H-6, H-8), 5.02 (dd, 1 H, partially obscured by water peak, H-1″), 4.02 (d, 1 H, $J_{5'',4''}$ 9.6 Hz, H-5″), 3.48–3.64 (m, 3 H, H-2″, H-3″, H-4″).

5. ^{1}H NMR (CD_3OD): δ 7.31 (AAB′B′ system, 2 H, $J_{3',2'}$ 8.9 Hz, H-3′, H-5′), 6.81 (AAB′B′ system, 2 H, H-2′, H-6′), 6.18 (d, 1 H, $J_{8,6}$ 3.3 Hz, H-8 or H-6), 6.17(d, 1 H, H-6 or H-8), 5.38 (dd, 1 H, $J_{2,3eq.}$ 2.6 Hz, $J_{2,3ax.}$ 12.9 Hz, H-2), 5.06 (dd, 1 H, $J_{1'',2''}$ 7.6 Hz, $J_{1'',3''}$ 3.6 Hz), 4.02 (d, 1 H, $J_{5'',4''}$ 9.6 Hz, H-5″), 3.46–3.62 (m, 3 H, H-2″, H-3″, H-4″), 3.16 (dd, 1 H, $J_{3ax.,3eq.}$ 17.1 Hz, H-3ax.), 2.73 (dd, 1 H, H-3eq.).

Direct-infusion ESI-MS

The complexes were formed in methanol by adding $CoBr_2$, a flavonoid glucuronide, and either 4,7-dpphen or 4,7-dmphen, all at 10 μM concentrations. Experiments were performed on an LCQ DUO quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA) with an ESI source. Solutions were introduced at a flow rate of 5 μl/min. The metal complexes were analyzed in the positive ion mode with a spray voltage of +5 kV and a heated capillary temperature of 150 °C. The gas flow rates, capillary voltage, and tube lens offset were optimized for maximum signal intensity on a daily basis. An ion injection time of 10 ms was used for full scan mass spectra and 50 ms was used for CID experiments. One hundred individual scans were averaged for each spectrum. The CID collision energies were converted from the normalized collision energy[36] values, given by the LCQ mass spectrometer, into absolute voltages applied to the ion trap during dissociation. Because the noncovalent complexes are relatively 'fragile', isolation windows of 3–5 m/z were required to obtain stable fragmentation spectra.[37] Such wide isolation windows have been reported for a variety of other noncovalent complexes examined by quadrupole ion trap MS.[38–40] Product ion abundances are reported relative to the most abundant ion in the spectrum, which is designated as 100%.

LC-MS analysis of urine samples

Samples of human urine collected after the consumption of grapefruit juice were obtained in a previous study.[16] Only samples from one human volunteer were reanalyzed. The 4.25-h timepoint was used because this was near the time of highest metabolite concentration excreted.[16] The frozen sample was thawed, and 400 μl of urine was added to 800 μl methanol and centrifuged for 5 min at 16 000 *g*. The supernatant was drawn off and evaporated with nitrogen. The sample was reconstituted in 1 ml of water with 0.05% formic acid (v/v). Solid phase extraction was performed using a C18 SepPak (Waters, Milford, MA). The SepPak was conditioned with methanol and 0.33% formic acid, followed by water and 0.33% formic acid. The urine extract was loaded, washed with 90 : 10 : 0.33 water/methanol/formic acid, and eluted with 2 ml 25 : 75 : 0.33 water/methanol/formic acid. Chromatography was performed on a Waters Alliance 2695 HPLC system. The stationary phase was a Waters Symmetry C18 column, 2.1 × 50 mm, 3.5 μm particles, with a matching guard column. An injection volume of 10 μl was used. The isocratic separation method used 30% water, 70% methanol, 0.05% formic acid, and a flow rate of 0.1 ml/min. The column effluent was sent to a UV detector operated at 280 nm prior to MS analysis. Postcolumn addition was then performed by mixing the effluent with a 5 μM methanolic solution of $CoBr_2$ and 4,7-dpphen flowing at 20 μl/min, which was added via a tee and controlled by a syringe pump. This mixture was introduced directly into the mass spectrometer, which was operated with a spray voltage of +4.5 kV and a capillary temperature of 200 °C. The gas flow rates, capillary voltage, and tube lens offset were optimized for the highest signal intensity of the complex of interest. The automatic gain control (AGC) settings were used with a target of 2×10^7 ions and 5-microscan averaging.

LC-MS analysis of plasma samples

The four quercetin glucuronides were spiked into 500 μl of rat plasma to achieve 1 μM concentrations. The analytes were stabilized as described by Day *et al.*[41] by the addition of 50 μl of 0.65 mM acetic acid and 10 μl of 50 mM ascorbic acid as an antioxidant. After gentle mixing, 1 ml of acetonitrile was added to precipitate the plasma proteins. The sample was vortexed for 1 min and centrifuged for 10 min at 16 000 *g*. The supernatant was collected and evaporated with nitrogen. The sample was reconstituted with 200 μl of water, 20 μl of 0.65 mM acetic acid, and 20 μl of 50 mM ascorbic acid. 10 μl of this sample was injected onto the HPLC column, using the same hardware and stationary phase as described for the urine samples. A gradient method was employed using water with 0.05% formic acid as mobile phase A and methanol with 0.05% formic acid as mobile phase B. The gradient started at 35% B and increased to 75% B over 20 min, then increased to 95% B over 1 min, followed by reequilibration. The flow rate was 0.1 ml/min. The UV signal was monitored at 370 nm. The postcolumn addition and MS conditions were the same as for the urine analysis.

RESULTS AND DISCUSSION

Direct-infusion ESI-MS/MS experiments

The availability of analytical quantities of flavonoid glucuronide standards is extremely limited, and compounds that cannot be purchased commercially must be synthesized. Some human metabolites of flavonoids occur in diglucuronidated form, but this study is limited to flavonoid

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monoglucuronides based on availability of standards. The four isomeric quercetin glucuronides were used to evaluate the efficacy of differentiation, while kaempferol-3-*O*-glucuronide, naringenin-7-*O*-glucuronide and baicalin were used to look for consistent indicators of glucuronidation at the 3 and 7 positions.

Before focusing on metal complexation, the ability to differentiate isomeric flavonoid glucuronides on the basis of tandem MS of the protonated or deprotonated species was evaluated. Because most of the standards were analyzed in their salt forms, these compounds provided very good signal intensity in the negative ESI mode. The MS/MS spectra of the $[L\text{-}H]^-$ ions, however, showed few distinguishing features (Table 1a). Three of the quercetin glucuronides yielded only one significant product ion (>5% relative abundance) resulting from the loss of the glucuronide moiety. It is impossible to determine the location of the glucuronide moiety on the basis of this evidence. Further stages of dissociation (MS^n) were not helpful. Quercetin-7-*O*-glucuronide provided additional product ions due to cross-ring cleavages[42] of the glucuronide moiety, such as $^{0,3}X^-$ and $^{0,2}X^-$, but these extra product ions were not observed in the CID spectra of the other flavonoid 7-*O*-glucuronides. Furthermore, naringenin-7-*O*-glucuronide and baicalin both displayed a prominent loss of the aglycon portion of the molecule, which did not occur for quercetin-7-*O*-glucuronide. Ideally, a distinctive fragmentation pattern would be indicative of a particular site of glucuronidation regardless of the aglycon portion (which can be identified by other MS strategies). CID of the deprotonated flavonoid glucuronides does not provide such consistent indicators. The protonated species were also examined, but they proved difficult to observe as two protons must be transferred to the negatively charged analytes to produce singly charged positive ions. The positively charged analytes could not be observed consistently even with the addition of acid, so the attempts to work with these species were abandoned.

Given the similarity between flavonoid glucosides and flavonoid glucuronides, it was hypothesized that a metal complexation approach[20–25,43] might be effective in determining the position of the glucuronide moiety, but the intrinsic negative charge on the flavonoid glucuronides proved to be a major obstacle in emulating these earlier methods. Divalent metal cations like Mn(II) and Mg(II), which had been so useful in previous applications,[24,25] did not form sufficiently abundant complexes with the analytes. This may be explained by the formation of neutral complexes of the type $[M(II)\ (L\text{-}H)_2]^0$, which are 'invisible' to the mass spectrometer. The metal ions Fe(III) and Al(III) were expected to form $[M(III)\ (L\text{-}H)_2]^+$ products, but these metals also failed to provide observable complexes. Monovalent metals such as Li and Na would form neutral adducts $[M(I)\ (L\text{-}H)]^0$ and so were not suitable candidates in the positive ESI mode, but the $[M(I)\ (L\text{-}H)_2]^-$ species were quite abundant in the negative ESI mode. However, identical fragmentation profiles were obtained upon CID of the isomeric species.

The formation of neutral flavonoid/metal complexes is a dead end for MS strategies, and one solution has been found involving the use of auxiliary ligands.[43] A neutral ligand replaces a negatively charged flavonoid conjugate in the complex, ensuring a net positive charge on the complex. The use of auxiliary ligands in flavonoid glycoside/metal complexes has been shown to improve the sensitivity of analysis,[43] and the ligand itself plays a vital role in determining how the complex will dissociate.[21] Phenanthroline-based ligands have been fruitful in the past, so they were selected for the present application. Two different auxiliary ligands, 4,7-dmphen and 4,7-dpphen, formed complexes with the flavonoid glucuronides and cobalt(II) while also allowing isomer differentiation by CID. Nickel(II) formed similar complexes, but the fragmentation results were rather less useful than with cobalt(II).

The best metal complexation mode found for differentiating the four quercetin monoglucuronide isomers involves the use of complexes of the form $[Co(II)\ (L\text{-}H)\ (4,7\text{-dmphen})]^+$. Dissociation of these complexes by CID results in four different fragmentation patterns, each with a unique set of product ions (Table 1b). The differentiation is based on the presence or absence of three key product ions of m/z 442 (loss of quercetin aglycon), m/z 536 (loss of the 4,7-dmphen molecule), and m/z 568 (loss of the glucuronide moiety). The quercetin-3-*O*-glucuronide complex dissociates to yield only one significant ion (>5% relative intensity) of m/z 536, in contrast to the other quercetin glucuronide complexes, for which the ion of m/z 568 is the most abundant product ion. The loss of the glucuronide moiety is the only substantial product ion yielded by the quercetin-4′-*O*-glucuronide complex, while the quercetin-3′-*O*-glucuronide complex and the quercetin-7-*O*-glucuronide complex yield small but significant losses of 4,7-dmphen and quercetin aglycon, respectively. It is hypothesized that the characteristic fragmentation patterns of these isomeric complexes are due to conformational differences that favor or disfavor the various dissociation pathways. This hypothesis is supported by the recent work of Clowers and Hill,[44] who used dual gate-ion mobility-quadrupole ion trap mass spectrometry to show that isomeric flavonoid glycosides complexed to various metal ions have different collision cross sections, an indication of conformational differences. Some complexes in that study assumed more than one stable conformation.

Energy variable CID experiments were undertaken to determine the dependence of these characteristic fragmentation pathways on collision energy (Fig. 3). It was found that the key product ions appear for all complexes beginning at around 1.0 V applied to the trap during dissociation, and these ions are fairly stable in their appearance until at least 1.6 V. The insensitivity of the fragmentation pathways to collision energy is beneficial in that there is no need for fine control of this parameter in order to obtain differentiation. In addition, these results are promising in terms of the ability to transfer this method to other tandem mass spectrometers that may be calibrated differently with respect to CID energies and performance.

While the $[Co(II)\ (L\text{-}H)\ (4,7\text{-dmphen})]^+$ complexes are extremely useful for differentiating the position of the glucuronide moiety of quercetin monoglucuronides, the

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Table 1. Product ions observed in direct-infusion MS/MS experiments

a. Deprotonated flavonoid glucuronides

CID (V)	Flavonoid glucuronide	Precursor ion	Product ions[a]			
			−GlcA	−Agl	$^{0,3}X^-$	$^{0,2}X^-$
0.48	Quercetin-3-*O*-glucuronide	477 (9)	301 (100)	–	–	–
0.48	Quercetin-3′-*O*-glucuronide	477 (13)	301 (100)	175 (1)	–	–
0.48	Quercetin-4′-*O*-glucuronide	477 (10)	301 (100)	175 (2)	–	–
0.53	Quercetin-7-*O*-glucuronide	477 (4)	301 (100)	175 (1)	373 (7)	343 (3)
0.56	Kaempferol-3-*O*-glucuronide	461 (8)	285 (100)	175 (2)	–	–
0.50	Naringenin-7-*O*-glucuronide	447 (14)	271 (100)	175 (95)	–	–
0.51	Baicalein-7-*O*-glucuronide	445 (14)	269 (100)	175 (25)	–	–

b. Flavonoid glucuronides in [Co(II) (L-H) (4,7-dmphen)]$^+$ complex

CID (V)	Flavonoid glucuronide (L)	Precursor ion	Product ions[a]		
			−GlcA	−Agl	−Aux
1.31	Quercetin-3-*O*-glucuronide	744 (0)	568 (1)	536 (100)	–
1.31	Quercetin-3′-*O*-glucuronide	744 (0)	568 (100)	536 (9)	442 (1)
1.31	Quercetin-4′-*O*-glucuronide	744 (0)	568 (100)	–	442 (1)
1.31	Quercetin-7-*O*-glucuronide	744 (0)	568 (100)	–	442 (6)
1.28	Naringenin-7-*O*-glucuronide	714 (0)	538 (100)	–	442 (21)
1.27	Baicalein-7-*O*-glucuronide	712 (0)	536 (100)	–	–

c. Flavonoid glucuronides in [Co(II) (L-H) (4,7-dmphen)$_2$]$^+$ complex

CID (V)	Flavonoid glucuronide (L)	Precursor ion	Product ions[a]				
			−GlcA	−Aux	−Agl	−(Aux + GlcA)	−(Aux + Agl)
1.56	Quercetin-3-*O*-glucuronide	952 (0)	776 (16)	744 (1)	650 (1)	568 (100)	–
1.56	Quercetin-3′-*O*-glucuronide	952 (0)	776 (4)	744 (100)	–	568 (78)	442 (1)
1.56	Quercetin-4′-*O*-glucuronide	952 (0)	776 (4)	744 (81)	–	568 (100)	442 (2)
1.56	Quercetin-7-*O*-glucuronide	952 (0)	776 (45)	744 (100)	650 (4)	568 (52)	442 (5)
1.54	Kaempferol-3-*O*-glucuronide	936 (0)	760 (2)	–	–	552 (100)	–
1.52	Naringenin-7-*O*-glucuronide	922 (0)	746 (100)	714 (63)	650 (13)	538 (71)	442 (13)
1.52	Baicalein-7-*O*-glucuronide	920 (0)	744 (11)	712 (28)	650 (4)	536 (100)	–

d. Flavonoid glucuronides in [Co(II) (L-H) (4,7-dpphen)$_2$]$^+$ complex

CID (V)	Flavonoid glucuronide (L)	Precursor ion	Product ions[a]						
			−H_2O	−GlcA	−Agl	−Aux	−(Aux + H_2O)	−(Aux + GlcA)	−(Aux + Agl)
1.67	Quercetin-3-*O*-glucuronide	1200 (0)	–	1024 (10)	898 (1)	868 (4)	–	692 (100)	–
1.67	Quercetin-3′-*O*-glucuronide	1200 (0)	1182 (1)	1024 (5)	–	868 (100)	850 (1)	692 (80)	566 (1)
1.67	Quercetin-4′-*O*-glucuronide	1200 (0)	1182 (2)	1024 (6)	898 (1)	868 (59)	850 (2)	692 (100)	566 (2)
1.67	Quercetin-7-*O*-glucuronide	1200 (0)	1182 (2)	1024 (40)	898 (7)	868 (100)	850 (3)	692 (63)	566 (5)
1.64	Naringenin-7-*O*-glucuronide	1170 (0)	1152 (6)	994 (100)	898 (19)	838 (53)	820 (3)	662 (82)	566 (10)
1.64	Baicalein-7-*O*-glucuronide	1168 (0)	1150 (1)	992 (12)	898 (6)	836 (17)	818 (1)	660 (100)	–

[a] Ions are tabulated by m/z ratio with the relative abundance in parentheses. Product ions below 1% relative abundance are not shown. Abbreviations used are as follows: −GlcA, loss of the glucuronide moiety; −Agl, loss of the aglycon portion; $^{0,3}X^-$, 0,3 cross-ring cleavage of the glucuronide moiety; $^{0,2}X^-$, 0,2 cross-ring cleavage of the glucuronide moiety; −Aux, loss of the auxiliary ligand.

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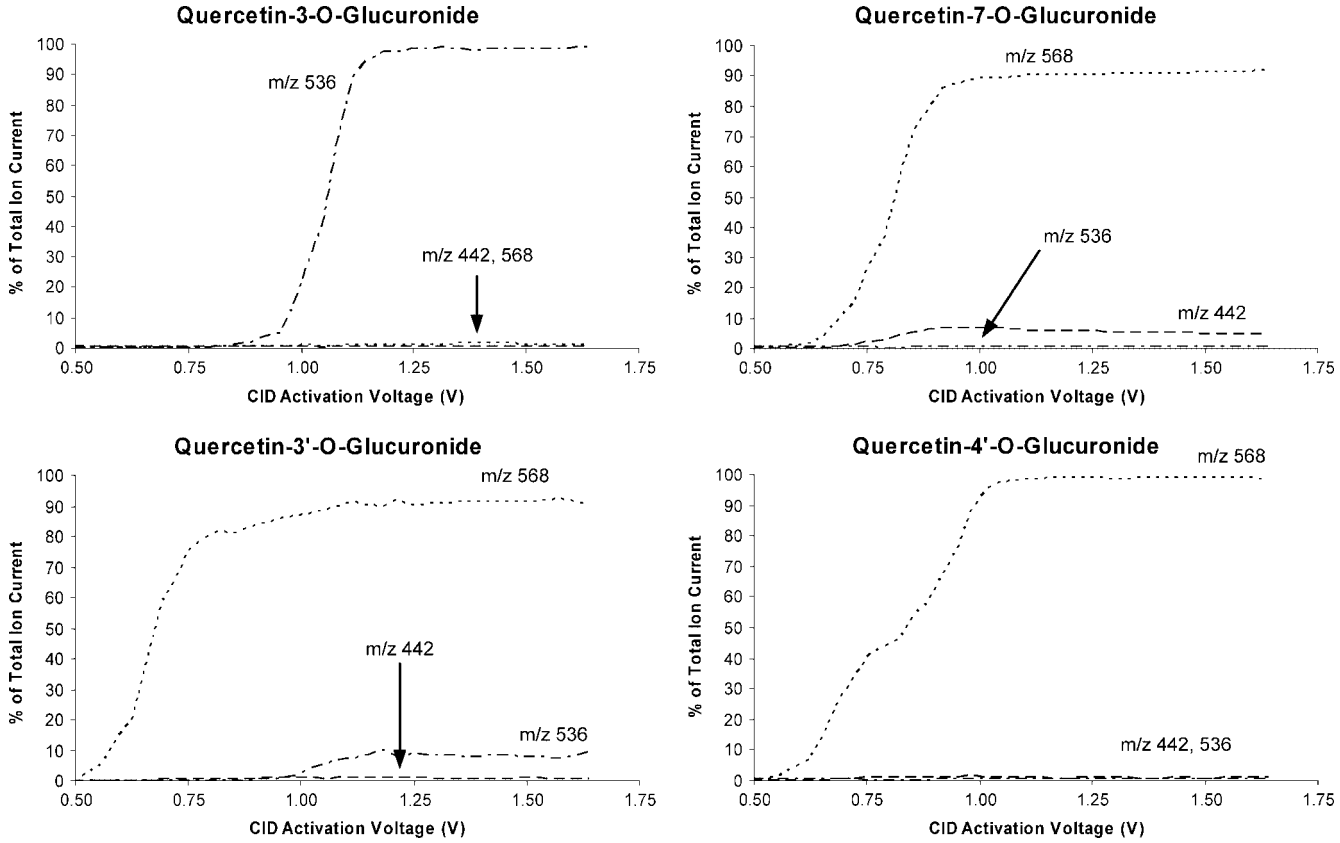

Figure 3. Energy variable CID results from [Co(II) (L-H) (4,7-dmphen)]$^+$ complexes involving quercetin glucuronides. The precursor ion and minor product ions are omitted. Fragments are identified as: *m/z* 568 (loss of the glucuronide moiety), *m/z* 536 (loss of 4,7-dmphen), *m/z* 442 (loss of the aglycon portion).

same patterns of product ions were not observed for compounds derived from other flavonoids. Instead, two other types of complexes were found that provide consistent fragments based on the glucuronidation position regardless of the type of flavonoid. For example, complexes of the form [Co(II) (L-H) (4,7-dmphen)$_2$]$^+$ yield consistent product ion profiles that reflect the position of glucuronidation for flavonols, flavones, and flavanones (Table 1c). The quercetin-3-*O*-glucuronide complex is differentiated from its isomers by the lack of a significant product ion corresponding to the loss of 4,7-dmphen (*m/z* 744). This ion is also absent from the CID spectrum of the kaempferol-3-*O*-glucuronide complex, suggesting a consistent indicator of glucuronidation at the 3 position. The quercetin-7-*O*-glucuronide complex is differentiated from its isomers as it is the only one that yields abundant product ions stemming from the individual losses of both 4,7-dmphen and of the glucuronide moiety. Only one of these two fragments appears with significant abundance in the MS/MS spectra of the other quercetin glucuronide complexes. Both product ions are also seen for the naringenin-7-*O*-glucuronide and the baicalin complexes. The presence of substantial amounts of both product ions (loss of 4,7-dmphen and loss of the glucuronide moiety) in the MS/MS spectra of only the complexes involving the flavonoid 7-*O*-glucuronides suggests a potential general method for identifying flavonols, flavones, and flavanones with a glucuronide moiety at position 7.

A third type of complex, [Co(II) (L-H) (4,7-dpphen)$_2$]$^+$, provides results similar to those obtained from [Co(II) (L-H) (4,7-dmphen)$_2$]$^+$, but is worth discussion because this complex was found to be the most adaptable to LC-MS analysis. The fragment ions are listed in Table 1d and the results of the energy variable CID experiments are shown in Fig. 4. It is seen that the [Co(II) (L-H) (4,7-dpphen)$_2$]$^+$ complex involving quercetin-7-*O*-glucuronide provides a unique signature of product ions (among the quercetin-based complexes), including significant losses of the glucuronide moiety and the 4,7-dpphen ligand, both individually and concurrently, as well as loss of the flavonoid aglycon (Fig. 4). Naringenin-7-*O*-glucuronide and baicalin also share these distinctive fragmentation characteristics (Table 1d). Quercetin-3-*O*-glucuronide can be differentiated by the lack of a highly abundant loss of 4,7-dpphen. Two other compounds, quercetin-3′-*O*-glucuronide and quercetin-4′-*O*-glucuronide, are differentiated on the basis of relative ion abundances. The two important products, the ions of *m/z* 692 and *m/z* 868, correspond to the loss of a 4,7-dpphen molecule either with or without the glucuronide moiety. In the range of 1.5–1.8 V of activation potential, the ion of *m/z* 868 is consistently the more abundant fragment of the quercetin-3′-*O*-glucuronide complex, while the ion of *m/z* 692 is the more abundant fragment of the quercetin-4′-*O*-glucuronide complex. Therefore, these two isomers can be confidently differentiated using a CID voltage in the range of 1.5–1.8 V.

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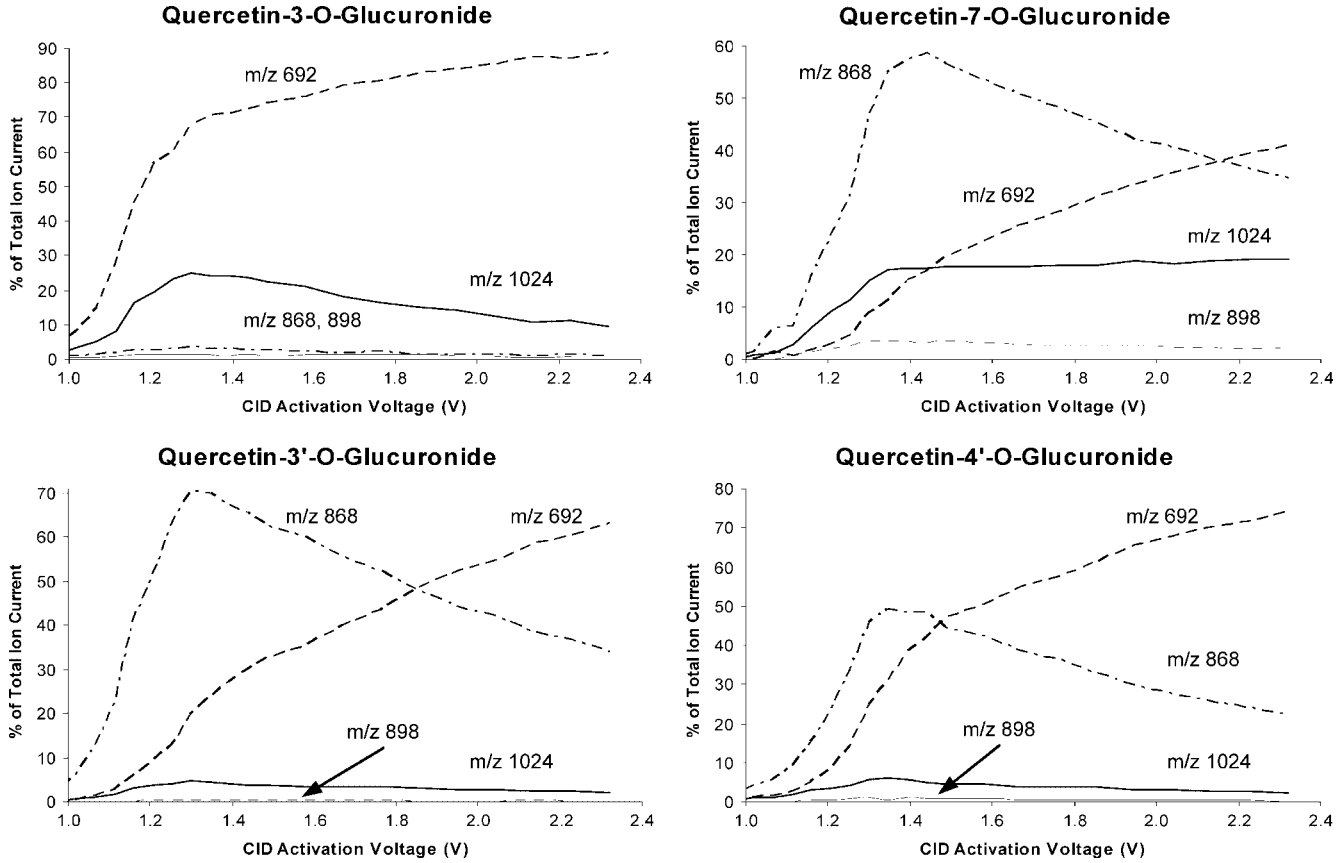

Figure 4. Energy variable CID results from [Co(II) (L-H) (4,7-dpphen)$_2$]$^+$ complexes involving quercetin glucuronides. The precursor ion and minor product ions are omitted. Fragments are identified as follows: *m/z* 1024, loss of the glucuronide moiety; *m/z* 898, loss of the aglycon portion; *m/z* 868, loss of 4,7-dpphen; *m/z* 692, loss of 4,7-dpphen and the glucuronide moiety.

LC-MS analysis of human urine

Urine samples were obtained from a metabolism study involving the consumption of grapefruit juice.[16] In that study, several glucuronidated and sulfated metabolites were partially identified by LC-MS on the basis of characteristic losses from the metabolites. However, the positions of glucuronidation could not be determined conclusively. One of these urine samples was reanalyzed using the new methods reported herein. The sample was prepared and analyzed as described in the Experimental section. Two naringenin glucuronides were found using LC-MS in the negative ESI mode by the *m/z* value of the deprotonated analyte (*m/z* 447) and the characteristic loss of the glucuronide moiety (−176 Da) upon dissociation.

When postcolumn complexation was employed, the analytes formed ample complexes of the type [Co(II) (L-H) (4,7-dpphen)$_2$]$^+$ of *m/z* 1170. Identification of the glucuronidation position was made by comparing the MS/MS data (Fig. 5) to those collected by direct infusion as described above. The fragmentation behavior of unknown **A** matched that of the naringenin-7-*O*-glucuronide complex. This assignment is consistent with the results of an alternate identification method based on retention time matching with an authenticated standard. Unknown **B** represents the more typical case where an authenticated standard is not available and the dissociation behavior of the complex is not known beforehand. This complex yields two significant product ions corresponding to the loss of 4,7-dpphen with and without the glucuronide moiety. This mirrors the behavior of the quercetin-3′-*O*-glucuronide and quercetin-4′-*O*-glucuronide complexes. As naringenin does not possess a hydroxyl group at the 3′ position, **B** was assigned as naringenin-4′-*O*-glucuronide. This assignment is in agreement with Abe *et al.*, who noted that naringenin-4′-*O*-glucuronide is retained longer by reversed-phase chromatography than the analogous 7-*O*-glucuronide.[45] The similarity of the fragmentation pathways suggests that consistent fragments found for model compounds may be extended to identify other flavonoid glucuronides without standards.

LC-MS analysis of rat plasma

The concentration of flavonoid glucuronides in human blood is typically between 0.1 to 2 μM following the consumption of flavonoid-rich foods.[6,41] Experiments were performed to determine whether metal complexation methods are effective at these low concentrations. A solution of the four quercetin glucuronide standards (1 μM each) was prepared and analyzed by LC-MS^n with postcolumn complexation. Each compound could be identified on the basis of the fragmentation of the [Co(II) (L-H) (4,7-dpphen)$_2$]$^+$ complexes (*m/z* 1200).

Having established that 1 μM concentrations can be analyzed in this way, the quercetin glucuronides were spiked into rat plasma at the 1 μM level, and were then extracted and analyzed as described in the Experimental section. All four

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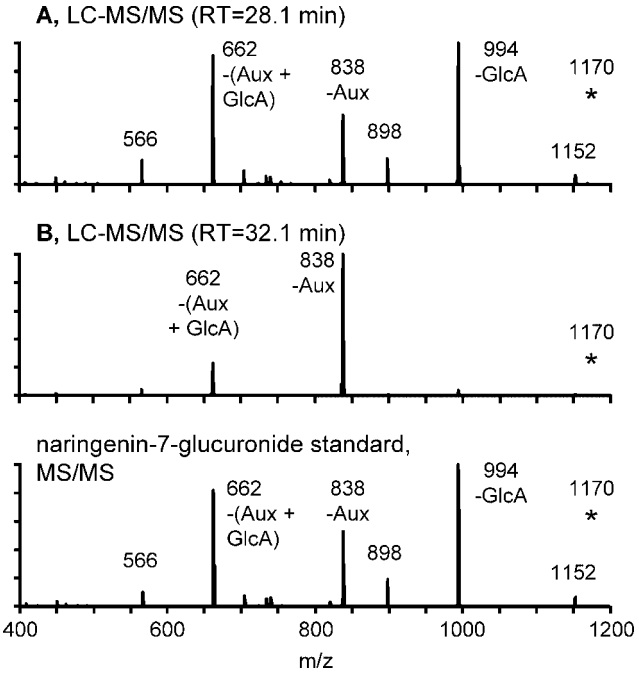

Figure 5. LC-MS/MS spectra of $[Co(II)\ (L\text{-}H)\ (4,7\text{-dpphen})_2]^+$ (m/z 1170) from human urine following consumption of grapefruit juice. A collision energy of 1.59 V was used. The direct-infusion MS/MS spectrum of the same complex involving a standard of naringenin-7-glucuronide, taken with a collision energy of 1.64 V, is also shown. The precursor ions are denoted by an asterisk (*). Fragments are abbreviated as follows: −GlcA, loss of the glucuronide moiety; −Aux, loss of 4,7-dpphen; 898, loss of the aglycon portion; 566, loss of 4,7-dpphen and the aglycon portion.

compounds could be observed and identified in this manner. On the basis of the similarities between these MS/MS results and those obtained by direct infusion, the four compounds were identified as follows: quercetin-7-*O*-glucuronide (retention time 10.5 min), quercetin-3-*O*-glucuronide (retention time 14.1 min), quercetin-4′-*O*-glucuronide, (retention time 17.4 min), and quercetin-3′-*O*-glucuronide (retention time 20.5 min). These results are promising in terms of using LC-MS methods to identify flavonoid glucuronides from blood samples obtained during *in vivo* studies.

CONCLUSION

We have described a versatile method for differentiating isomeric flavonoid glucuronides by mass spectrometry. Complexes are formed by mixing a flavonoid glucuronide, $CoBr_2$, and an auxiliary ligand (either 4,7-dmphen or 4,7-dpphen) in solution. Performing ESI-MS/MS on these complexes leads to characteristic product ions that allow isomer differentiation and determination of the position of the glucuronide moiety. Four isomeric quercetin glucuronides were differentiated on the basis of unique product ion profiles obtained from these complexes. Glucuronide derivatives of kaempferol, naringenin, and baicalein formed similar complexes that fragmented in an analogous manner to the quercetin glucuronides, allowing the location of the glucuronide moiety to also be determined for these compounds. These examples suggest the possibility that glucuronide derivatives of other flavonols, flavones, and flavanones may be characterized in the same way. This method may be performed by direct-infusion ESI-MS/MS (using pure flavonoid glucuronides) or by LC-MS/MS of mixtures using postcolumn complexation. The LC-MS/MS method was used to support the isomeric assignments of two naringenin glucuronides in human urine following the consumption of grapefruit juice. The method was also proven to be sensitive enough for *in vivo* plasma analysis at realistic analyte concentrations. It is envisioned that the methods described here would be used in concert with other methods to achieve complete qualitative analysis of flavonoid metabolites.

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