APPLICATION NOTE

Simultaneous Quantification of Multiple Licorice Flavonoids in Rat Plasma

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Flavonoids are important naturally occurring polyphenols with antioxidant properties. In this study, we report the development of a liquid chromatography tandem mass spectrometry (LC–MS/MS)–based method capable of simultaneously quantifying multiple active licorice flavonoids (including liquiritin apioside, liquiritin, liquiritigenin, isoliquiritin apioside, isoliquiritin, and isoliquiritigenin) in plasma. Electrospray ionization was used to efficiently generate precursor deprotonated molecules of all the analytes and the $[M–H]^-$ ions were used to produce characteristic product ions for MS/MS analysis. We found that inclusion of a very low concentration of $HCOONH_4$ (0.01‰) in the LC mobile phase dramatically improved the detection limit for the tested flavonoids and decreased the interference by matrix effects, which have been referred to as "LC-electrolyte effects." Liquid–liquid extraction with ethyl acetate was effective for isolation of all the analytes and resulted in the lowest matrix effects of several tested sample cleanup methods. This bioanalytical method showed good linearity between 0.32 ng/mL and 1 μg/mL analyte in 50-μL plasma samples. The accuracy and precision at different analyte concentrations varied from 85 to 110% and from 0.8 to 8.8%, respectively. Finally, we demonstrated the applicability of this method in a pilot pharmacokinetic study of rats receiving an oral dose of Xiaochaihu-tang, an important Chinese herbal remedy for chronic hepatitis. The use of a low concentration of $HCOONH_4$ in the LC mobile phase could be used to improve LC–mass spectroscopy- or LC–MS/MS-based methods. (J Am Soc Mass Spectrom 2007, 18, 778–782) © 2007 American Society for Mass Spectrometry

Unlike synthetic drugs, herbal drugs contain a number of chemical constituents that may have pharmacological activities or therapeutic effects. Because pharmacokinetic information is needed for the design and interpretation of toxicity and clinical studies, monitoring the blood levels of known active constituents, representative markers, or major chemical constituents in botanical drug products is strongly encouraged by the U.S. Food and Drug Administration (FDA) [1]. Pharmacokinetic measurements for natural products often involve the assay of multiple analytes from complex biological samples, such as plasma and urine. These assays are complicated and the reliability of the results may not be adequate.

Liquid chromatography tandem mass spectrometry (LC–MS/MS) is the current method of choice for pharmacokinetic studies, although adverse matrix effects can lead to analysis errors and failures [2, 3]. For pharmacokinetic studies, LC–MS/MS-based protocols for analysis of multiple analytes in complex biomatrices should include: (1) high ionization efficiency for all analytes without cross-interference; (2) efficient extraction and recovery of all analytes from the biological sample; (3) relative ease of use, including simplicity, flexibility, and high throughput; and (4) a lack of adverse matrix effects for all analytes without a loss of sensitivity, specificity, or accuracy.

As part of our ongoing studies of Xiaochaihu-tang [4, 5], a Chinese herbal remedy used to treat chronic hepatitis, we developed an LC–MS/MS-based method for the simultaneous quantification of several licorice flavonoids in plasma samples, including three flavonones [liquiritin apioside (LQA; $C_{26}H_{30}O_{13}$; MW = 550), liquiritin (LQN; $C_{21}H_{22}O_9$; MW = 418), and liquiritigenin (LQG; $C_{15}H_{12}O_4$; MW = 256)] and three chalcones [isoliquiritin apioside (ILA; $C_{26}H_{30}O_{13}$; MW = 550), isoliquiritin (ILN; $C_{21}H_{22}O_9$; MW = 418), and isoliquiritigenin (ILG; $C_{15}H_{12}O_4$; MW = 256]. Recent studies have indicated that these antioxidant flavonoids have pharmacological effects [6]. We report the development of an LC–MS/MS-based method that can simultaneously quantify six licorice flavonoids in rat plasma. An earlier liquid chromatography–ultraviolet (LC–UV)–based method for quantification of liquiritigenin in urine was reported [7], but it may not be

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suitable for plasma pharmacokinetic studies because of poor sensitivity.

Experimental

Chemicals and Materials

The reference standards liquiritin apioside (≥99%), liquiritin (≥99%), liquiritigenin (≥99%), isoliquiritin apioside (≥99%), and isoliquiritin (≥99%) were obtained from the Phytochemistry Department of this institute. Isoliquiritigenin (>98%) was purchased from Extrasynthèse (Genay, France).

The herb components of Xiaochaihu-tang tea (XCH-tea) consisted of *Radix Bupleuri* (364 g), *Radix Scutellariae* (273 g), *Rhizoma Pinelliae* (273 g), *Radix Ginseng* (273 g), *Radix Glycyrrhizae* (182 g), *Rhizoma Zingiberis Recens* (273 g), and *Fructus Jujubae* (364 g). The herbal materials (2 kg total) were coarsely ground, mixed, soaked in water for 1 h, and extracted twice with water (4 and 3 L) at 80 °C for 0.5 h each. The extracts were combined (~4.6 L), filtered, and concentrated to 3 L to yield XCH-tea. This preparation contained 1472, 116, 8.24, 185, 18.0, and 4.05 μg/mL of LQA, LQN, LQG, ILA, ILN, and ILG, respectively, and was stored at −20 °C until use.

Preparation of Plasma Samples

Rat plasma (50 μL) was acidified with 10 μL of 0.25 M HCl, mixed with 1 mL EtOAc, vortexed, and centrifuged. The upper organic phase (850 μL) was evaporated to dryness. The residue was reconstituted in 50 μL of CH_3CN/H_2O (24:76, vol/vol, containing 0.01‰ $HCOONH_4$) and centrifuged. The supernatant (10 μL) was used for LC–MS/MS analysis.

LC–MS/MS

The LC–MS/MS system consisted of a TSQ Quantum triple-quadrupole mass spectrometer interfaced by an electrospray ionization (ESI) probe with a Surveyor LC system controlled by Xcalibur software (ThermoElectron, San Jose, CA). Chromatographic separations were achieved on a 5-μm Kromasil C_{18} column (50 × 2.0-mm internal diameter; Bohus, Sweden; 30 °C). The LC mobile phases consisted of CH_3CN/H_2O (10:490, vol/vol, containing 0.01‰ $HCOONH_4$; solvent A) and CH_3CN/H_2O (450:50, vol/vol, containing 0.01‰ $HCOONH_4$; solvent B). The LC gradient was delivered at 0.2 mL/min and consisted of an initial linear increase from 22 to 72% B over 3 min, followed by a return to 22% B over 0.1 min, and maintenance at 22% for 4.9 min. Infusion experiments were carried out to optimize the negative ion ESI-MS/MS instrument parameters for maximal generation of deprotonated molecules and efficient production of characteristic fragment ions for each analyte. The precursor-to-product ion pairs (m/z 549 → 255 for LQA and ILA, m/z 417 → 255 for LQN and ILN, and m/z 255 → 119 for LQG and ILG) were monitored.

Construction of Standard Curves

Plasma from untreated rats was spiked with 1 μg/mL of the six analytes and then serially diluted with additional plasma to 200, 40, 8, 1.6, and 0.32 ng/mL. Standard curves were constructed using weighted ($1/X$) linear regression of the peak areas of the analyte (Y) versus the corresponding plasma concentrations of the analyte (X; ng/mL).

Assessment of Matrix Effects and Extraction Efficiency

Matrix effects, including both absolute and relative matrixèeffectsè[8],èandèextractionèefficienciesèwereèexamined in quintuplicate by comparing the peak areas of analytes between three different sample sets. In set 1, analytes were dissolved in matrix component-free reconstitution solvent. In set 2, analytes were added into five different lots of preextracted plasma from untreated rats. In set 3, analytes were added to plasma from untreated plasma and then extracted. The absolute matrix effect, relative matrix effect, and extraction efficiency were calculated as follows:

Absolute matrix effect

$$= (\text{Mean peak area})_{\text{set 2}} / (\text{Mean peak area})_{\text{set 1}}$$

Relative matrix effect

$$= (\text{RSD of peak area})_{\text{set 2}} - (\text{RSD of peak area})_{\text{set 1}}$$

Extraction efficiency

$$= (\text{Mean peak area})_{\text{set 3}} / (\text{Mean peak area})_{\text{set 2}}$$

Method Validation

Assays were validated according to the U.S. FDA guidanceè onè bioanalyticalè methodè validationè [9].è Assay selectivity was assessed by monitoring the ion pairs of the six analytes for the appearance of peaks in plasma from five untreated rats. Cross-interference among analytes was established by comparing the peak area obtained from the reconstitution solvent containing one of the tested flavonoids at 8 ng/mL and the other five at 1 μg/mL each versus the peak area obtained from solutions containing only the tested flavonoids at 8 ng/mL. Analyte stability was determined at two concentrations (200 and 8 ng/mL) under conditions mimicking the entire assay process. To assess the accuracy and precision of the assay, plasma samples (50 μL) containing concentrations of 1000, 40, or 0.32 ng/mL of the six analytes were analyzed using the above protocol, and the measured concentrations were calculated from their linear regression equations and compared to the nominal concentrations.

Results and Discussion

LC–MS/MS Detection of the Tested Licorice Flavonoids

In initial experiments, we developed an efficient ionization method for detecting the tested flavonoids. In the positive-ion ESI experiment, the peak attributed to the protonated molecule $[M+H]^+$ was detected in all spectra. Except for LQA and LQN, which showed higher levels of $[M+NH_4]^+$ ammonium adducts, the $[M+H]^+$ peak was the highest. In-source product ions corresponding to the loss of a monosaccharide or disaccharide residue were detected for the four glycosyl flavonoids, with the flavonone glycosides LQA and LQN showing more extensive fragmentation than the chalcone glycosides ILA and ILN. In the negative-ion ESI experiment, the peak attributed to the deprotonated molecule $[M–H]^-$ predominated in all spectra. The high $[M–H]^-$ ion abundance and the low tendency to form undesirable ions (i.e., in-source fragment ions and adducts) in the negative ESI mode resulted in a higher ionization efficiency. Atmospheric pressure chemical ionization (APCI) gave a low ionization efficiency for the tested glycosides resulting from a high incidence of in-source fragmentation, which substantially reduced the formation of $[M+H]^+$ ions in the positive-ion mode or $[M–H]^-$ ions in the negative-ion mode. Thus, APCI appeared to be unsuitable for the purposes of this study.

Based on our comparison of ionization methods, we chose negative-ion ESI for generation of the precursor deprotonated molecules $[M–H]^-$. In the MS/MS spectra, the most abundant ions at *m/z* 255 were ascribed to the loss of a glucose residue (LQN and ILN) or an apiosyl-glucose one (LQA and ILA). The main product ions for the aglycones LQG and ILG were at *m/z* 119 along with a less abundant product ion at *m/z* 135 ($^{1,3}B$ and $^{1,3}A$, respectively, using the nomenclature by Domon and Costello [10]).

Inclusion of a high concentration (10–100 mM) of mobile phase additive was previously shown to reduce the ESI response [11]. In this study, we found that decreasing the concentration of $HCOONH_4$ in the CH_3CN/H_2O mobile phase from 1.00‰ (16 mM) to 0.01‰ (0.16 mM) resulted in a dramatic increase in signal intensity, with only a slight change in the pH (Figure 1). For all analytes, there was a uniform reaction of the signal intensity to the change in the $HCOONH_4$ concentration. The highest signal intensity was achieved when the concentration of $HCOONH_4$ in the mobile phase was 0.01‰ (3.0- to 5.1-fold higher than that in electrolyte-free mobile phase). This concentration (0.01‰ or 0.16 mM) is much lower than the usual concentration (5–50 mM) of electrolyte used in the mobile phase.

We next investigated whether the benefit of the low concentration of mobile phase additive might be offset by a decreased chromatographic performance and/or undesirable matrix effects. We found that the concentration of $HCOONH_4$ had only negligible effects on the chromatographic peak widths, peak asymmetry, and peak retention times. Furthermore, a mobile phase containing 0.01‰ $HCOONH_4$ resulted in minimal matrix effects for all of the tested flavonoids. In particular, the extent and variability of the matrix effects for LQA, LQN, and ILG were significantly reduced compared to the results when an $HCOONH_4$-free mobile phase was used (Figure 2). These results indicated that negative-ion ESI with an unusually low concentration of mobile phase additive (0.01‰) was the most effective protocol for the needs of this study.

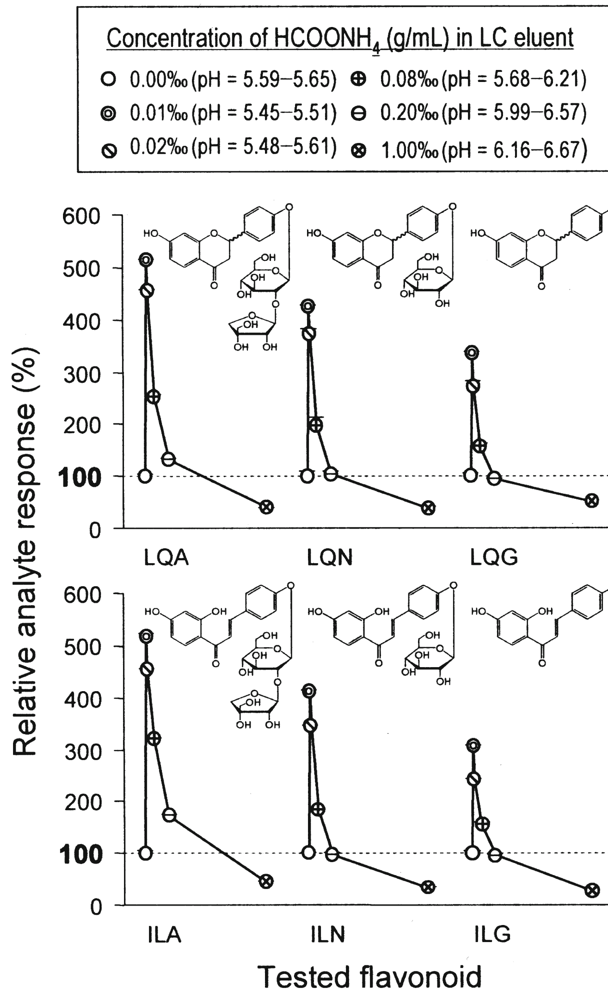

Figure 1. Effect of the $HCOONH_4$ concentration in LC mobile phase on the signal intensity of the tested licorice flavonoids in negative-ion ESI mode. The response (the peak area) is given relative to that using the electrolyte-free mobile phase.

Preparation of Plasma Samples

It is inconclusive which is the most effective sample cleanup method for overcoming matrix effect problems [2, 3]. Here, we compared three common sample preparation techniques (liquid–liquid extraction, protein precipitation, and solid-phase extraction) for their abilities to extract multiple analytes and for their associated matrix effects. We found that both the aglycone and

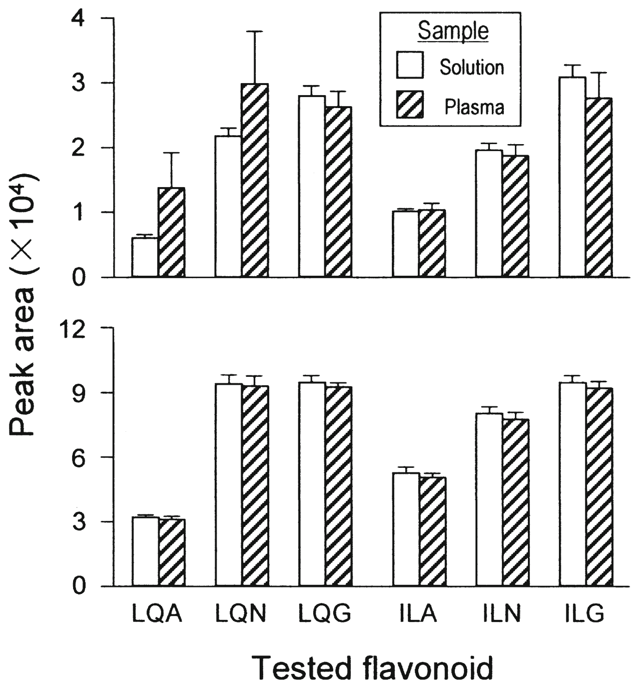

Figure 2. Matrix effects on the formation of $[M–H]^-$ ions from the tested licorice flavonoids in negative-ion ESI mode using an LC mobile phase consisting of pure CH_3CN/H_2O (top panel) or CH_3CN/H_2O containing 0.01‰ $HCOONH_4$ (bottom panel). Plasma samples from five untreated rats were extracted with EtOAc and then spiked with 8 ng/mL of the tested flavonoids. The chromatographic retention times were 2.0, 2.3, 4.4, 3.8, 4.0, and 5.5 min for LQA, LQN, LQG, ILA, ILN, and ILG, respectively.

glycosyl flavonoids could be efficiently extracted from plasma using protein precipitation with CH_3CN or MeOH at a precipitant-to-plasma volume ratio of 2:1 or using solid-phase extraction on Oasis® HLB C_{18} cartridges (Waters, Milford, MA). The extraction efficiencies for the protein precipitation methods were > 66%, whereas those for the solid-phase extraction methods were 90–125%. Of the tested liquid–liquid extraction solvents (EtOAc, MTBE, $CHCl_3$, CH_2Cl_2, ether, and *n*-hexane), only EtOAc is effective for all of the analytes, giving extraction efficiencies > 50% in all cases.

The EtOAc-based liquid–liquid extraction method was found to result in weaker matrix effects than the protein precipitation and solid-phase extraction methods. Therefore, we used the EtOAc-based liquid–liquid extraction method for subsequent experiments. Except for ILG, which gave a small ion suppression (−7.6%), the EtOAc-extracted samples showed slight ion enhancements (3.0–7.7%) resulting from the extractable plasma components. The relative matrix effects were also small for all of the analytes (4.6–10.0%), whereas the respective relative standard deviation (RSD) values of the repeated peak area measurements from control samples dissolved in reconstitution solvent were between 2.0 and 6.9%. For the protein precipitation method, CH_3CN outperformed MeOH. Using protein precipitation with CH_3CN, the absolute matrix effects were −16.5–15.2% for the analytes, except LQN, which enhanced ionization by 31.1%. The associated relative matrix effects were small (1.0–11.0%). Protein precipitation of the plasma samples with MeOH resulted in pronounced absolute matrix effects for some analytes (such as ion enhancements of 90.8% for LQA and 29.9% for LQN and ion suppressions of −16.1% for LQG and −24.8% for ILA). When solid-phase extraction was used, we also observed high degrees of absolute matrix effects (−19.2–13.4%) and relative matrix effects (5.7–23.9%).

Validation of the Analytical Method

All of the tested licorice flavonoids were stable under conditions mimicking those in real plasma samples. Chromatographic resolution was achieved in 8 min, allowing separation of isomers (that is, LQA versus ILA, LQN versus ILN, and LQG versus ILG). No coelution of endogenous compounds was observed at retention times corresponding to the tested flavonoids, demonstrating that the method had good specificity. Moreover, we found no evidence of cross-interference among the analytes.

Matrix-matched standard curves of the peak area of a given analyte (Y) versus the nominal plasma concentration (X; ng/mL) were linear for concentrations between 0.32 ng/ml and 1 μg/mL, with correlation coefficients > 0.99. The typical regression equations for LQA, LQN, LQG, ILA, ILN, and ILG were $Y = 2186X - 699$, $Y = 7695X - 193$, $Y = 8313X + 1427$, $Y = 3523X + 274$, $Y = 6405X + 943$, and $Y = 8060X + 535$, respectively. The RSD values for the concentrations measured on the same day ($n = 5$) and those measured on different days ($n = 5$) for plasma quality control standards never exceeded 8.8% at any of the tested concentrations, indicating good assay precision. The assay accuracy ranged from 85 to 110% for all of the analytes. The method provided a lower limit of quantification of 3.2 pg on-column for all of the analytes.

To demonstrate the applicability of our method, we applied it to a pilot pharmacokinetic study of Xiaochaihu-tang in rats. As shown in Figure 3, both the aglycone and glycosyl licorice flavonoids could be detected in plasma up to 24 h after administration of XCH-tea. Thus, the assay can be used to assess systemic exposure of rats to licorice flavonoids.

Use of a stable isotope-labeled internal standard (IS) in monoanalyte bioassays usually yields precise and accurate results from LC–MS/MS-based assays. Although structural analogs of the analyte are often used as the IS, the nature and degree of matrix effects may be very different for the analyte and the IS analogue. For multianalyte bioassays, generation of a stable isotope-labeled IS for each analyte is expensive, time-consuming, and impractical. Furthermore, our recent work indicated that using a single isotope-labeled IS does not adequately resolve matrix effect problems in multianalyte bioassays [12]. In this study, we initially tested a number of potential IS candidates, but the tested compounds did not allow us to determine the relative matrix effects on the quantifica-

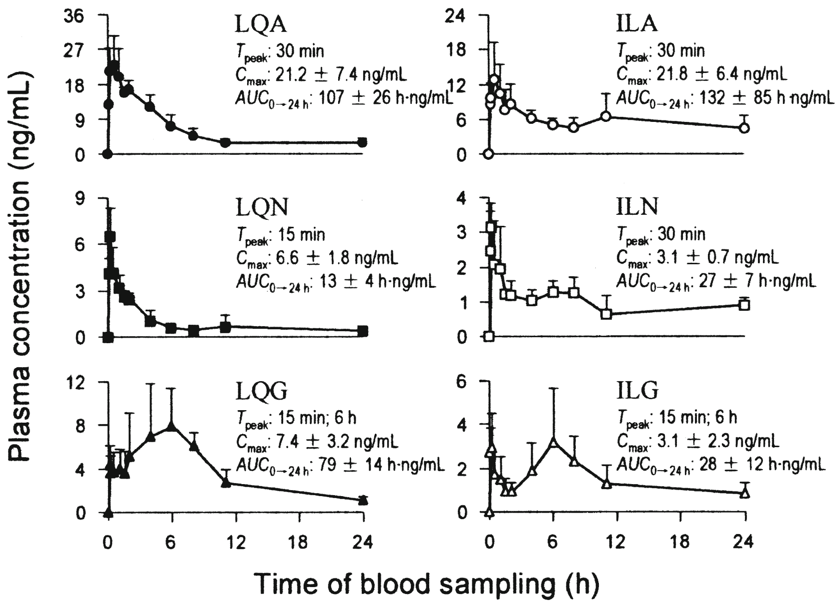

Figure 3. Plots of the plasma concentrations of tested licorice flavonoids versus the time of blood sampling. Sprague–Dawley rats were given a single oral dose of 15 mL/kg XCH-tea, and blood samples were taken after 0, 5, 15, 30 min and 1, 1.5, 2, 4, 6, 8, 11, and 24 h.

tion of the analytes. Instead, we determined the six plasma licorice flavonoids in parallel in the absence of an IS. The preceding validation results indicated that the analysis could be performed reliably in the absence of an IS, arising from an "LC-electrolyte effect" that minimizes the matrix effects, and using matrix-matched calibration curves to calculate the analyte concentrations of the real samples in each run.

Conclusion

In the present study, we developed a sensitive method for quantification of multiple licorice flavonoids in plasma. We optimized this method for maximal extraction/detection performance and minimal matrix effects. The LC mobile phase in the optimized method contained a much lower concentration of $HCOONH_4$ than commonly used (0.01‰), which dramatically increased the ESI efficiency of the analytes and decreased the matrix effects. These have been referred to as "LC-electrolyte effects." This is a particularly important finding because increased MS detection sensitivity often relies on the design of new ion sources or mass spectrometers. Thus, we developed a non-instrumental technique for improving the sensitivity limit of MS detection. Although no IS was used, our validation studies consistently demonstrated that the bioanalytical results were accurate, reproducible, and reliable. Furthermore, the applicability of the method was demonstrated in a pilot pharmacokinetic study in rats.

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