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Post-column mobile phase adjustment: A strategy to eliminate the contradiction between liquid chromatography and mass spectrometry in the determination of flavonoids in rat plasma

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ABSTRACT

Flavonoids are a group of important naturally occurring polyphenolic compounds with a wide range of biological effects. In this study, a sensitive liquid chromatography tandem mass spectrometry method was developed to simultaneously determine multiple active flavonoids, including quercetin (Que), kaempferol (Kae), apigenin (Api), isorhamnetin (Iso), luteolin (Lut), and naringenin (Nar), in rat plasma. To achieve a satisfied peak shape and LC separation, formic acid with the concentration between 0.05 and 0.2%, or in some case 5%, was generally used to acidify the LC mobile phase in reported studies. Here we found that even 0.05% formic acid could lead to strong mass signal suppression, and the absence of formic acid could reverse the signal suppression but cause serious peak tailing. There is an irreconcilable contradiction between liquid chromatography (LC) and mass spectrometry (MS). In order to simultaneously satisfy LC and MS, LC mobile phase with 0.00075% formic acid and post column mobile phase adjustment with 0.0677% ammonium solution in isopropanol were applied. Compared with the conventional method with mobile phase containing 0.05% formic acid, the mass signal response of Que, Kae, Api, Iso, Lut, Nar, and Oka increased 26.2, 18.6, 13.6, 23.5, 17.5, 15.6 and 15.4 fold, respectively. In addition, the post column mobile phase addition exhibited the better peak shape for the reduction of analytes longitudinal diffusion. The method has been fully validated according to FDA guidelines within the linear range between 0.328 ng mL^{-1} and 168 ng mL^{-1}, and successfully applied to a pilot pharmacokinetic study of rats after administering 5.43 g kg^{-1} Pollen of *Brassica campestris*.

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1. Introduction

Flavonoids are a group of polyphenolic compounds diverse in chemical structure and characteristics. They occur naturally in flowers [1], nuts [2], fruit [3], vegetables [4], and are part of the human diet. Flavonoids have been proved to possess a wide range of biological effects, including anti-inflammatory [5], antioxidation [6], anticancer [7], antibacterial [8], and vasodilatory actions [9]. In addition to these beneficial aspects, flavonoids also could cause damage to the body such as food–drug [10] and herb–drug [11] interaction mediated by CYP enzymes and drug transporters. Monitoring the systematic exposure of active flavonoids will help us for the dosing design and interpretation of therapeutic effect and toxicity.

The pharmacokinetic study of active flavonoids always involved in multiply trace amount of analytes and large number of complex biological samples. Thus, the method, with high throughput, sensitivity and selectivity, is required. Since the pK_a of flavonoids is about 6.5, acidification of the mobile phase is essential to achieve good peak shape and resolution. Generally, 0.05–0.2%, or in some case 5% of formic acid [12–15] was applied for acidification. However, we found that the mass signal intensity was extremely suppressed even with 0.05% formic acid in LC elution. Removing formic acid significantly increased the mass signal response but led to serious peak tailing. Thus, there was an irreconcilable contradiction between liquid chromatography (LC) and mass spectrometry (MS) in the quantification of flavonoids. In this study, post-column mobile phase adjustment was applied to obtain satisfied chromatographic

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resolution and mass spectrometry response. In addition, the strategy not only eliminates the contradiction but also brings extra benefits on the peak shape and mass signal intensity.

As part of our ongoing studies of Pollen of *Brassica campestris*, a famous healthy food and phytomedicine for the treatment of benign prostatic hyperplasia (BPH) in China [16], a LC–MS/MS method was developed for the simultaneous quantification of six active flavonoids (Fig. 1), including quercetin (Que) [17–19], kaempferol (Kae) [20,21], apigenin (Api) [22,23], isorhamnetin (Iso) [24,25], luteolin (Lut)[26,27] and naringenin (Nar) [28,29], in rat plasma. The post-column mobile phase adjustment system was detailed discussed in this study, which not only provides the principle for better use of the system but also improved our knowledge on the utilization of negative ESI source.

2. Experimental

2.1. Chemicals and materials

Pollen of *Brassica campestris* were provided by Zhejiang Conba Pharmaceutical Co., Ltd (Hangzhou, China). Quercetin, kaempferol, apigenin, isorhamnetin, and luteolin (purity ≥ 98%) were received from the National Institute for Food and Drug control of China (Beijing, China). Naringenin (Purity ≥ 98%) were purchased from Aladdin (Shanghai, China). Okanin (purity ≥ 98%) was prepared in our lab. HPLC-grade formic acid was provided by Roe Scientific Inc. (DE, USA). HPLC-grade acetonitrile (ACN) was from Tedia Company, Inc. (Fairfield, USA). HPLC-grade methanol (MeOH), ethyl acetate, and isopropanol (IPA) were provided by Merck & Co., Inc. (Darmstadt, Germany). Analytical-grade ammonia solution and ascorbic acid (purity > 99%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Distilled water was from Watson Group Ltd. (Hong Kong, China).

2.2. Instrumentation

The Agilent 1290 liquid chromatography system consisted of a G4220A binary pump, a G1330B column oven, and a G4226A autosampler (Agilent, CA, USA). Mass spectrometric detection was performed on an Agilent 6460 triple quadrupole instrument (Agilent, CA, USA) equipped with an ESI source. Agilent MassHunter software (version B.04.01, Agilent) was used for data acquisition and processing. Post-column mobile phase adjustment was made after the analytical column, using the micro flow pump (Unimicro, CA, USA), via a T-connection (Agilent, CA, USA).

2.3. Chromatographic conditions

The chromatographic separation was performed on a poroshell 120 SB C_{18} column (2.7 μm, 2.1 mm × 50 mm, Agilent, USA) maintained at 30 °C with a gradient elution (0–1.50 min, 10–45% of B; 1.50–3.50 min, 45–55% of B; 3.50–3.70 min, 55–72% of B, 3.70 – 3.71 min, 72–10% of B, 3.71–5.0 min, 10% of B) at 0.36 mL min^{-1}, where mobile phase A and B were water and MeOH with 0.00075% formic acid, respectively. The post-column mobile phase adjustment was done with the addition of IPA with 0.0667% ammonia solution at the flow rate of 0.24 mL min^{-1}.

2.4. Mass spectrometric conditions

Mass spectrometric analysis was performed using an ESI source in negative ion mode. Quantification was obtained using multiple reaction monitoring (MRM) mode at m/z transitions of 315→300 for Iso, 301→179 for Que, 285→285 for Kae, 269→117 for Api, 285→133 for Lut, 271→151 for Nar, and 287→135 for Oka (IS), respectively. The mass spectrometer conditions were optimized and set as follows: gas temperature 325 °C, gas flow 5 L min^{-1}, nebulizer 45 psi, capillary voltage 3500 V, sheath gas temperature 350 °C and sheath gas flow 11 L min^{-1}. Fragmentor voltage was set at 144, 150, 165, 155, 170, 130 and 110 V for Iso, Que, Kae, Api, Lut, Nar and IS, respectively. Collision energy was 16, 13, 20, 38, 34, 14 and 24 v for Iso, Que, Kae, Api, Lut, Nar and IS, respectively.

2.5. Preparation of standard solutions

Stock solutions were prepared by dissolving the reference standards (Iso: 1 mg mL^{-1}, Que: 1 mg mL^{-1}, Kae: 1 mg mL^{-1}, Api: 0.8 mg mL^{-1}, Lut: 1 mg mL^{-1}, Nar: 1 mg mL^{-1}) in MeOH: DMSO (3/2, v/v). Mixed working solutions were prepared at concentrations of 0.00781, 0.01563, 0.0125, 0.25, 0.5, 1.0, 2.0, 3.2, and 4.0 μg mL^{-1} via addition of proper volume of individual stocking solution in tube and diluting with MeOH: water (4:1, v/v).

2.6. Preparation of calibration standards and quality control samples

Calibration standards were prepared by spiking the pooled rat plasma (6 lots) with 2.1 μL corresponding working solution to yield final concentrations of 0.328, 0.656, 5.250, 10.50, 21.0, 42.0, 84.0, and 168.0 ng mL^{-1} for the 6 analytes. Quality control (QC) samples containing the 6 analytes (0.328, 0.656, 10.50, and 134.4 ng mL^{-1}) were also prepared as the above method.

2.7. Sample preparation

A 20 μL aliquot of 0.1 M sodium acetate buffer (pH = 5) containing glucuronidase (913 units mL^{-1}) and sulfatase (44.65 units mL^{-1}) and 5 μL ascorbic acid (103.49 mg mL^{-1}) were added to plasma sample (50 μL), and the sample was incubated in a sealed vial for 5 h at 37 °C with continuous shaking. After hydrolysis, 100 μL 0.1% formic acid, 25 μL IS solution (20 ng mL^{-1} Oka in ethyl acetate) were added. The mixture was extracted with 975 μL of ethyl acetate by vortexing for 6 min. After centrifugation at 9300 × g for 3 min, 650 μL of the organic phase was transferred to another vial and evaporated to dryness at 37 °C in the vacuum concentrator systems (LABCONCO, MO, USA), then the residue was reconstituted in 60 μL of MeOH: water (1:1, v/v) containing 6.46 mg mL^{-1} ascorbic acid. The aliquot of 5 μL was injected into the LC–MS/MS system.

2.8. Method validation

The assay was validated according to FDA guidelines for bioanalytical method validation [30]. The selectivity was tested by analyzing blank plasma from six rats and spiked rat plasma at the lower limit of quantification (LLOQ). The calibration curves were constructed from the peak area ratio of each analyte to Oka against the nominal concentrations using 8 levels non-zero standards and a linearly weighed ($1/x^2$) least squares regression model. LLOQ were determined by analyzing five replicates of quality control (QC) samples at 0.328 ng mL^{-1}. Intra-batch and inter-batch precision and accuracy were assessed at four different concentrations (0.328, 0.656, 10.50, and 134.4 ng mL^{-1}) for each analyte. Relative standard deviation (RSD %) and relative error (RE %) were calculated for precision and accuracy, respectively. The extraction recovery was determined at three levels (0.656, 10.50, and 134.4 ng mL^{-1}) by comparing the peak area of MER (samples spiked with analytes prior to extraction) with MEX-1 (pooled blank plasma samples spiked post-extraction). The matrix effect was evaluated at the concentration of 0.656 ng mL^{-1} by comparing the peak area of MEX-2 (six lots rat plasma spiked post-extraction individually) with MEP (reconstitution solvent containing equal concentration). Plasma sample stability, such as keeping at room temperature, 37 °C,

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