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Application of ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry to identify curcumin metabolites produced by human intestinal bacteria

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ABSTRACT

Curcumin, a yellow pigment derived from the rhizomes of *Curcuma longa* Linn, is a natural antioxidant that exhibits a variety of pharmacological activities and therapeutic properties. However, as curcumin is generally conjugated when absorbed through the intestine, free curcumin is present at extremely low levels in the body. Thus, curcumin metabolites are presumed to be responsible for curcumin bioactivity. In this study, we describe a strategy using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF MS) with automated data analysis software (MetaboLynx^{XS}) for rapid analysis of the metabolic profile of curcumin in human intestinal flora. The results show that curcumin undergoes extensive phase I and phase II metabolism. A total of 23 curcumin metabolites were detected and identified *in vitro*. Furthermore, we identified a number of novel metabolic pathways of curcumin in the human intestinal microflora system.

1. Introduction

Curcumin, a widely used natural food product in curry powder and food coloring, is the major bioactive component in turmeric (*Curcuma longa* L.) [1]. Curcumin is a potential therapeutic agent for the treatment of a wide variety of human diseases, including cancer [2,3], immune deficiencies [4], cardiovascular health [5], Alzheimer's [6], diabetes [7], arthritis [8], and Crohn's disease [9], due to its beneficial activities and lack of toxicity. However, despite its potential health benefits, the use of curcumin is limited by poor aqueous solubility, intense staining color, and extremely low oral bioavailability. Poor systemic bioavailability of curcumin is attributable, at least in part, to metabolism [10]. In previous studies, various methods, such as HPLC, GC–MS, and HPLC–MS/MS, were used to analyze curcumin metabolism *in vitro* and *in vivo*. These studies revealed that curcumin undergoes extensive metabolism through reduction and conjugation [11–16]. Early studies suggested that, following oral administration, curcumin undergoes metabolic O-conjugation to curcumin glucuronide and curcumin sulfate, and bioreduction to tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol in rats and mice *in vivo* [17–19], in suspensions of human and rat hepatocytes [17], and in human and rat intestinal cytosol [12]. Further, data suggest that the intestinal tract plays an important role in the metabolic disposition of curcumin [12].

A considerable portion of ingested curcumin reaches the cecum and colon, where a large population of indigenous bacteria exists. Metabolites of curcumin produced by human intestinal bacteria have hitherto not been identified. Understanding the metabolic pathways involved in its biotransformation is crucial for the design of derivatives with increased anti-cancer activity and/or decreased toxicity.

Ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF MS) is a selective, sensitive, and high-resolution technique that can be used to analyze and identify metabolites with mass accuracy. Here, we describe an approach for determining curcumin metabolites produced by human intestinal microflora using UPLC–Q-TOF MS. These experiments will ultimately aid in rationalize drug design for future chemopreventives derived from curcumin.

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2. Experimental

2.1. Materials and methods

Curcumin (>90% purity) was obtained from Zhejiang Institute for Food and Drug Control (Hangzhou, China). Calcium chloride, Epsom salt, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium bicarbonate, and sodium chloride were supplied by Sinopharm Chemical Reagent (Beijing, China). Tryptone, yeast extract, and cysteine hydrochlorate were obtained from BBI (Canada). HPLC grade acetonitrile, methanol, and formic acid were purchased from TEDIA Inc. (Fairfield, USA). Ultra-pure water (18.2 MΩ) was obtained from an ELGA-Purelab Ultra system (HighWycombe, UK). All other chemicals were from standard commercial sources and were of the highest quality.

2.2. Collection and preparation of intestinal bacteria

Fresh human fecal samples were obtained from one healthy male volunteer who had not taken any medicine in three months and avoided alcohol and food rich in curcumin for 48 h before collection. The fecal sample (4.0 g) was weighed, suspended in a centrifuge tube with 20 mL of sterile physiological saline, and homogenized by a vortex-mixer. The mixture was centrifuged at 2000 × *g* for 10 min. The supernatant was added to 18 volumes of anaerobic medium and cultivated overnight in anaerobic incubator. The anaerobic medium was modified peptone-yeast extract (PY) medium contained 20 mg/mL tryptone, 10 mg/mL yeast extract, 0.5 mg/mL cysteine hydrochlorate, and 40 mL/L VPI salt solution, pH 7.2. The VPI salt solution consisted of 0.2 mg/mL calcium chloride, 0.2 mg/mL Epsom salt, 1 mg/mL dipotassium hydrogen phosphate, 1 mg/mL potassium dihydrogen phosphate, 10 mg/mL sodium bicarbonate, and 2 mg/mL sodium chloride. All procedures were performed at 4 °C.

2.3. Incubation of curcumin in human intestinal bacteria

All media and buffers required for fermentation were left in the anaerobic chamber (BioMérieux, Marcy l'Etoile, France) containing two small bags of BioMérieux GENbox Anaer (bioMérieux Marcy l'Etoile, France) maintained at 5% CO_2, 10% H_2, and 85% N_2 for at least 24 h before the experiment. The atmosphere of the jar containing the fecal sample was made anaerobic by using two packages of GENbox Anaer before being placed in the incubator (Thermo, USA) at 37 °C.

Curcumin (100 μM) was added to the intestinal bacteria culture overnight. Incubation in the absence of intestinal bacteria was used as a negative control. The incubation mixture was incubated for either 0 or 24 h, collected, and frozen immediately to stop the reaction. The sample was centrifuged (13,000 rpm, 10 min), and the supernatants were analyzed by UPLC–Q-TOF MS.

2.4. UPLC and MS conditions

The SYNAPT G2-S Q-TOF mass spectrometer (Waters, Manchester, UK) was connected to the Acquity UPLC system (Waters, Milford, MA, USA) *via* an electrospray ionization (ESI) interface. Chromatography was performed on the UPLC with a conditioned autosampler at 4 °C, using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters, Milford, MA, USA). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), and a gradient elution of 0–4 min, 10–40% B; 4–8 min, 40% B; 8–13 min, 40–85% B; 10–13 min, 100% B; 13.1–15 min, 10% B was used for the equilibration of the column. The flow rate was 0.4 mL/min, and the injection volume was 5 μL. The ESI source was operated in the negative ionization mode. Typical source conditions for maximum intensity of precursor ions were as follows: capillary voltage, 2.5 kV; sample cone, 30 V; source temperature, 120 °C; desolvation temperature 500 °C; cone gas flow rate 50 L/h; desolvation gas (N_2) flow rate 800 L/h. Leucine-enkephalin was used as the lock mass, generating an $[M-H]^-$ ion (*m*/*z* 554.2615) to ensure accuracy during the MS analysis. Data were collected in centroid mode with the lockspray interval set at 10 s, and were averaged over 3 scans. The MS^E experiment in two scan functions was carried out as follows: function 1 (low energy): 100–1000 mass-scan range; 0.2 s scan time; 0.015 s inter-scan time; 2 eV collision energy; function 2 (high energy): 100–1000 mass-scan range; 0.2 s scan time; 0.015 s inter-scan time; collision energy ramp of 10–40 eV. Acquiring data in this manner allowed us to collect information on intact precursor ions and fragment ions.

2.5. Data processing

Data processing was performed using a MetaboLynx^{XS} program (Waters Corp., Milford, MA, USA), which uses an expected metabolite list based on potential biotransformation reactions [20]. This software automates the detection and identification of metabolites by comparing the sample with the control to eliminate endogenous interfering ions from the complex matrices. Data analysis with Metabolynx^{XS} was performed in three steps. Firstly, the acquired data were processed using a user-defined parameter file to generate a preliminary report file which involved an automated comparison of analyte LC/MS chromatograms with appropriate control samples. Secondly, the report is displayed in the browser, and the output is refined by a variety of data filters. Finally, a large number of peaks were obtained, which must be inspected manually to determine whether they were likely to be compound-related metabolites. A threshold of 5 mDa was set as a limit to filter the processed data for the calculation of possible elemental composition. The parent compound was added to the window of the expected metabolites list, and the mass window was set to 10 mDa. Unexpected metabolite chromatograms were created over the full mass acquisition range in the mass window (10 mDa). The peak detection was accomplished using the Apex-Track algorithm, and the threshold of the peak area was set to 0.5 units.

3. Results and discussion

3.1. UPLC–Q-TOF MS analysis of curcumin

The chromatographic and MS fragmentation behaviors of the parent drug were investigated by UPLC–Q-TOF MS. The protonated curcumin (M0 at *m*/*z* 367) was eluted at a retention time of 9.25 min. As illustrated in Fig. 1A, the spectrum of *m*/*z* 367.1186 generated fragment ions at 217, 173, 149, and 134. The fragment ion at *m*/*z* 217 was generated by the cleavage of the C=C double bond, followed by the loss of CH_3 (15 Da). A previous report showed that curcumin underwent tautomerization between keto (form A) and 3-keto-5-enol (form B) [21]. The *m*/*z* 173 fragment was derived from the loss of $C_{10}H_{10}O_4$ from the protonated molecular ion (form B). The *m*/*z* 149 fragmented was formed through the loss of 219 ($C_{12}H_{10}O_4$) from the protonated molecular ion. This fragment ion could further lose CH_3 (15 Da) to give the ion at *m*/*z* 134. Based on these results, the fragmentation pathway of curcumin was proposed as shown in Fig. 1B. These fragment ions were used as references to aid in the interpretation of metabolite fragment ions, as well as to examine the high resolution and mass accuracy of the instrument.