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Deciphering the influence of column chemistry and mass spectrometry settings for the analyses of geometrical isomers of L-chicoric acid

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ABSTRACT

Resolving the chemo-diversity of plant extract samples is an essential step for in-depth analyses of natural products which often exhibit promising biological activities. One of the challenges in this endeavor has been the confident differentiation of geometrical isomers. In this study, we investigated these aspects in chromatography (column chemistry and mobile phase composition) and mass spectrometry settings with regards to better differentiation of geometrical isomers. A standard of a hydroxycinnamic acid (HCA) derivative, L-chicoric acid (L-CA) – a di-acylated caffeoyltartaric acid ester found in a number of plant families – was used. Geometrical isomers of L-CA were formed by exposing the compound to ultraviolet (UV) radiation, to mimic the natural environment. The high performance liquid chromatography photo-diode array (HPLC-PDA) and ultra-high performance liquid chromatography mass spectrometry (UHPLC–MS) platforms were used to analyze the *trans* and *cis* geometrical isomers of L-CA. The HPLC-PDA results confirmed the generation of two *cis* geometrical isomers following UV exposure of the authentic *trans*-L-CA standard. Furthermore, the HPLC-PDA analyses demonstrated that the changes in both column chemistry (reverse-phase: C_{18}, biphenyl, phenyl-hexyl and pentafluorophenyl propyl) and mobile phase composition (aqueous acetonitrile and aqueous methanol) affect the chromatographic elution profiles of the L-CA isomers. The MS results, on the other hand, revealed undisputed fragmentation differences between the geometrical isomers of L-CA. Thus, this study demonstrates that the identification of the L-CA isomers can be achieved more efficiently and confidently with good chromatography coupled to well-optimized mass spectrometry conditions, a requirement which has been proven impossible with other types of HCA derivatives. Moreover, differences in the binding modes of L-CA geometrical isomers to the HIV type 1 integrase enzyme were observed, suggesting a synergistic anti-HIV-1 activity of these isomers.

1. Introduction

Plants provide a unique diversity of secondary metabolites (natural products) that can be exploited for the discovery of a broad range of health benefits and other useful applications: new drugs, nutraceuticals and cosmetic ingredients, just to name a few. Diving into the natural products' realm remains, however, strongly limited by the inherent chemo-diversity of the plant extracts, the equipment, and particularly by the expertise and methodologies applied for confident isolation and structural elucidation of metabolites of interest. One of these natural products with potent biological properties is L-chicoric acid (2,3-dicaffeoyl-L-tartaric acid, L-CA), which is a di-acylated hydroxycinnamoyl tartaric acid ester, naturally found in a variety of plant species [1–5]. This compound is known to possess biological properties of great importance [5], for instance such as anti-human immunodeficiency virus (HIV) type 1 DNA integrase activity [6]. The biological activity of L-CA is reportedly related to its isomers [7] which is an important observation as L-CA, being a hydroxycinnamic acid (HCA) derivative, forms geometrical isomers when exposed to ultraviolet (UV) radiation, a natural phenomenon in the plants' environment [1]. Here, Healy et al. [7] observed that the orientation of the *cis*-isomers of L-CA

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led to binding inside the catalytic pocket of the HIV-1 integrase enzyme. This HIV-1 INT is a key enzyme responsible for the integration of viral DNA into the host genome [7,8] and, as such, is a good target for drug design against HIV infection. Interestingly, various hydroxyl-cinnamic acid derivatives have been shown to possess anti-HIV-1 INT enzyme activity, including 3,5-dicaffeoylquinic acid (3,5-diCQA) which we recently showed to be slightly affected by the *trans-cis* isomerization [8]. Moreover, a biosynthetic analogue of L-CA, was also found to exhibit potent anti-HIV-1 INT activity compared to the naturally occurring 3,5-diCQA counterpart [9].

In-depth analyses of the plant extracts containing L-CA metabolites would imperatively require thorough analytical methodologies, that would enable the confident differential identification of the isomers for clarity on the etiology of the reported potent biological properties. This points to ongoing efforts in natural product research, geared towards a continuum of advancing analytical understanding and development of approaches that would aid in the correct and differential identification of metabolites of interest. For instance, mass spectrometry methods targeted at distinguishing between positional isomers of HCA derivatives have been developed [10]. In contrast, very little has been done for geometrical isomers [11]. Thus, from an analytical perspective, the differential characterization of geometrical isomers is still an undisputed challenge. Clifford et al. suggested chromatographic elution order to be a reliable approach to differentiate UV-generated geometrical isomers of hydroxycinnamoyl-quinic acids (chlorogenic acids, CGAs) [12]. Notably, CGAs are structurally related to hydroxycinnamoyl-tartaric acids such as L-CA, as these esters result from an esterification reaction between HCA derivatives and the respective organic acids, quinic acid and tartaric acid. However, geometrical isomers of CGAs seemingly produce similar fragmentation patterns when analysed by an ion trap (IT)-MS system [12,13]. This poses a challenge in the discriminative identification of these geometrical isomers, particularly from complex plant extracts, as a *cis* isomer can be mistaken for a *trans* counterpart and *vice-versa*.

In this study, by revisiting and exploring the chromatographic separation space, insightful information and perspectives can be gained, which can aid in designing optimal methods for differential characterization of natural products or plant metabolites. Accordingly, we investigated some common aspects of chromatography to determine the influence of column chemistry and mobile phase composition on the separation of UV-generated L-CA geometrical isomers. Here four different reverse-phase (RP) column matrices (C_{18}, biphenyl, phenyl-hexyl and pentafluorophenyl propyl (PFPP)) and two mobile phase compositions (aqueous methanol and aqueous acetonitrile) were investigated. Moreover, simple MS conditions (*i.e.* collision energy) were altered to achieve differentially informative fragmentation data of L-CA molecules. Lastly, using molecular docking methods, differences in the binding modes of UV-induced geometrical isomers of L-CA on the HIV-1 INT were examined.

2. Materials and methods

2.1. Materials

An authentic standard of *trans*-L-chicoric acid (*trans*-L-CA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Analytical grade methanol and acetonitrile were obtained from Romil (Cambridge, UK). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chromatographic separation was achieved using three Kinetex analytical columns: C_{18} (4.6 × 150 mm, with particle size of 5 μm; Phenomenex, Torrence, CA, USA), biphenyl (4.6 × 100 mm, with particle size of 5 μm; Phenomenex, Torrence, CA, USA) and phenyl-hexyl (4.6 × 100 mm, with particle size of 5 μm; Phenomenex, Torrence, CA, USA) and two Ultra analytical columns: pentafluorophenyl propyl (PFPP) (2.1 × 100 mm, with particle size of 3 μm; Restek, Bellefonte, PA, USA) and biphenyl column (2.1 × 100 mm, with particle size of 3 μm; Restek, Bellefonte, PA, USA).

2.2. Methods

2.2.1. UV-irradiation

A 1 mg/mL solution of *trans*-L-CA was prepared with 100% methanol. The solution was placed in a UV-light-box (Spectroline, Model CM-10) operating at 254 nm short wavelength for 2 h. Aliquots (100 μL) were taken at 0 h (before irradiation) and at 1 h and 2 h post irradiation. The aliquots were diluted 10 × with 100% methanol and subjected to HPLC-PDA and UHPLC–MS analyses.

2.2.2. HPLC-PDA parameters

The HPLC system used was a Shimadzu SCL-10A VP (Kyoto, Japan), equipped with a PDA controlled by Shimadzu VP software v. 5.31. Column oven temperature was set at 30 °C. The injection volume was 5 μL. A binary solvent mixture was used, consisting of MilliQ water (eluent A) containing 0.1% formic acid and methanol or acetonitrile (eluent B) containing 0.1% formic acid. The initial conditions were 10% B at a flow rate of 0.8 mL/min and were maintained for 3 min followed by multiple gradients to 65% B at 53 mins, the conditions were kept constant for 3 min and then changed to the initial conditions (10% B) after 3 min; followed by a 3 min isocratic wash at 10% B to re-equilibrate column. The total chromatographic run time was 62 min. The PDA detector scanning range was set from 220 to 400 nm and the chromatograms were processed at 330 nm.

2.2.3. Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLC–MS) parameters

The samples were analysed on a Nexera UHPLC (Shimadzu Corporation, Kyoto, Japan) interfaced to an electrospray- triple quadrupole (ESI-QqQ) mass spectrometer and fitted with an Ultra reverse-phase biphenyl column (Restek, USA). The injection volume was 5 μL and the column oven temperature was maintained at 30 °C. A binary solvent mixture was used consisting of MilliQ water (eluent A) containing 0.1% formic acid and methanol (eluent B) containing 0.1% formic acid. A flow rate of 0.4 mL/min was used for analyte separation. The initial conditions were 10% B and were maintained for 3 min followed by multiple gradients to 65% B at 53 min. The conditions were kept constant for 3 min and then changed to the initial conditions (10% B) after 3 min; followed by a 3 min isocratic wash at 10% B to re-equilibrate column. The total chromatographic run time was 62 mins. An ultraviolet (UV) fixed wavelength detector was used at 330 nm.

The mass spectrometer settings used were: interface voltage was set at 3.5 kV (in negative ESI mode), 35 eV collision energy, 250 °C source temperature, nitrogen was used as the drying gas at the flow rate of 15.00 L/min and as a nebulizing gas at 3.00 L/min flow rate, argon was used as a collision gas with a pressure of approximately 230 kPa in the collision cell. A mass range of 100–600 Da was used in order to include the precursor ion at *m/z* 473 and the product ion at *m/z* 311. The scan time was 1 s. To validate the consistency of the fragmentation patterns obtained between the L-CA geometrical isomers, different collision energy (CE) settings (5, 10, 15, 20, 25, 30 and 35 eV) were utilized [14] to achieve useful MS fragmentation data. In addition, alternating interface voltages (1.5, 2.5, 2.5, 3.5 and 4.5 kV) were also explored at a collision energy of 25 eV as this collision energy was not too harsh and showed the presence of the product ion at *m/z* 311.