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Journal of Chromatography B, 852 (2007) 398–402
JOURNAL OF CHROMATOGRAPHY B
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Synthesis of safflomide and its HPLC measurement in mouse plasma after oral administration

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Received 16 October 2006; accepted 29 January 2007
Available online 8 February 2007

Abstract

Safflomide (*N*-caffeoyltryptamine) is a compound belonging to a group of phenylpropanoid amides found in plants. In this study, safflomide was chemically synthesized and confirmed by LC–MS, LC–MS/MS and NMR spectroscopic methods, and a high-performance liquid chromatography (HPLC) method was developed for quantifying safflomide in biological samples. The synthesis was simple, and the yield of safflomide was greater than 50%. Using the synthesized safflomide as a standard, HPLC separation was performed on a Nova-Pak C18 column using an isocratic buffer, and the separation was detected using a coulometric electrochemical detector. The detection of safflomide yielded an excellent peak resolution at the retention time of 21 min, and the lower limit of the detection was as little as 100 fmol. Using this HPLC method, the plasma concentrations of safflomide were determined in mouse blood, collected at 5, 10, 15, 20, 25, 30, and 35 min following its oral administrations (1 and 3 mg/30 g body weight). This HPLC method standardized with safflomide is the first reported method able to quantify the compound in standard and plasma samples with good detection limit and consistent reproducibility.

Keywords: *N*-Caffeoyltryptamine; Safflomide; HPLC; Oral administration; Mouse plasma; Bioavailability

1. Introduction

Safflomide (*N*-caffeoyltryptamine) is a phenylpropanoid amide produced in plants via forming an amide bond between the carboxyl group of caffeic acid and the amine group of tryptamine [1–5]. *N*-Caffeoyltryptamine and its analogues are found in numerous plants, including *Coffea canephora*, *Theobroma cacao*, and *Carthamus tinctorius* [4–8]. Recent studies indicate that the compounds may contain biological activities, implicated in preventing and/or treating pathophysiological conditions from human chronic diseases such as inflammation and atherosclerosis [9–12]. However, only little is known about the effect of *N*-caffeoyltryptamine on the human diseases. Furthermore, bioavailability of the compound is nearly unknown, making it difficult to accurately propose and/or assess its effects in humans. Therefore, in this study, safflomide (Fig. 1) was first synthesized chemically, because the compound is not yet available commercially, and a high-performance liquid chromatography (HPLC) method was developed using the synthesized safflomide as a standard. Since the developed HPLC method provides the excellent peak resolution of *N*-caffeoyltryptamine with high sensitivity and reliability, the method was used for measuring the concentrations of safflomide in mouse plasma following oral administrations.

2. Experimental

2.1. Materials and reagents

Tryptamine, caffeic acid, dichloromethane, 1,3-diisopropylcarbodiimide, and Supelco C18 (3 μm particle size, 250 mm × 3.0 mm) reversed-phase column were purchased from Sigma Chemical Co. (St. Louis, MO). Nova-Pak C18 (3 μm particle size, 2.1 mm × 150 mm) was purchased from Waters (Milford, MA)

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doi:10.1016/j.jchromb.2007.01.047

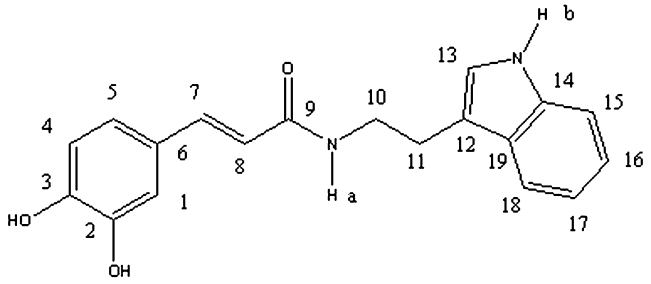

Fig. 1. Chemical structure of safflomide (*N*-caffeoyltryptamine).

2.2. Chemical synthesis of safflomide

Synthesis was performed as described previously [13,14]. Briefly, caffeic acid (1 g) was dissolved in dichloromethane (5 mL) and converted to the symmetrical anhydride with 1,3-diisopropylcarbodiimide (1 mL). Tryptamine (1 g) was added to the reaction mixture, and then stirred gently for 3 h at room temperatures. The synthesized products were recovered by precipitating with distilled water and washing with ethylacetate and 5% $NaHCO_3$ to remove the unreacted starting materials [14]. The recovered products were further purified by HPLC (Waters, Milford, MA) for the experiments.

2.3. LC–MS, LC–MS/MS, and NMR analyses of safflomide

The synthesized products were prepared for LC–MS and LC–MS/MS analyses, as described previously [15]. LC–MS was performed with an Agilent 1100 HPLC system coupled with LCQ class ion-trap mass spectrometer using electrospray ionization (ESI) in positive ion mode. A Supelco C18 (3 μm particle size, 250 mm × 3.0 mm) reversed-phase column operated at 25 °C at a flow rate of 0.25 ml/min. The mobile phases were water and acetonitrile containing 0.1% formic acid as described previously [15]. For the confirmation of safflomide (*N*-caffeoyltryptamine), LC–MS/MS was also performed to monitor the fragments of safflomide from *m/z* 85 to 350, with the following conditions: sheath gas flow rate, 70 (arbitrary units); aux gas flow rate, 10 (arbitrary units); spray voltage, 4.50 kV; heated capillary temperature, 220 °C; capillary voltage, −4.0 V; tube lens offset, 25 V. The collision energy for the LC–MS/MS was set at 30%.

For NMR experiments, the sample was prepared by dissolving safflomide (25 mg) in d_6-DMSO (0.75 mL). 1H, 13C, COSY, HSQC and HMBC spectra were acquired at ambient temperature on the JEOL BCX-400 NMR spectrometer operating 400 MHz for 1H and 100 MHz for 13C. Chemical shifts were referenced to DMSO (2.50 ppm for 1H, 39.5 ppm for 13C).

2.4. HPLC method

N-Caffeoyltryptamine standards were prepared in the range from 0.1 to 10 μM in 40% methanol. A 2.1 mm × 150 mm i.d. Nova-Pak C18 (Waters, Milford, MA) was used as the stationary phase to analyze *N*-caffeoyltryptamine, and an isocratic buffer of 50 mM NaH_2PO_4 (pH 4.3) containing 40% methanol was used at 1 mL/min as the mobile phase for the HPLC analyses. *N*-Caffeoyltryptamine in the samples was injected by an autosampler into Alliance 2690 HPLC system (Waters, Milford, MA), and was detected by CoulArray electrochemical detector with four electrode channels (ESA, Chelmsford, MA). The amount of *N*-caffeoyltryptamine in the samples was quantified by CoulArray electrochemical detector software (version 1.0).

2.5. Plasma sample preparation

For measuring the concentrations of *N*-caffeoyltryptamine in plasma samples, Swiss Webster mice 20–25 weeks old (Charles River, Wilmington, MA) were used for collecting blood. All animal procedures were performed according to an animal protocol approved by Beltsville Agricultural Research Center (BARC). Mice were fed AIN-76A purified diet that provided the recommended allowance of all nutrients required for maintaining optimal health, but lacking *N*-caffeoyltryptamine tested in the study; the diet was analyzed by HPLC, ensuring that the compound was not in the diet. Mice in the experimental trial were divided into three groups (control and two oral dosing groups), and each group consisted of five mice. For the two oral dosing groups, safflomide (1 and 3 mg/30 g body weight) was administrated into the animal using a dosing needle. Because safflomide samples were prepared in 10% ethanol (100 μL), the same liquid vehicle was also administrated into the mice in the control group using a dosing needle. Blood was collected via the tail bleeding technique at 5, 10, 15, 20, 25, 30, and 35 min after the oral dosing. Whole blood was centrifuged at 2000 × *g* for 10 min, and the plasma was saved for experiments. To extract safflomide from plasma samples, the plasma samples (60 μL) were precipitated with methanol (40 μL) and centrifuged at 14,000 × *g* for 10 min. The final supernatant was used for the assay.

2.6. Data analyses

All statistic analyses were performed with the StatView program of SAS institute Inc. (Cary, NC, USA). The linearity of regression line was determined by the correlation coefficient (*R*). Data points in all figures represent the mean ± SD of more than three samples.

3. Results and discussion

3.1. Chemical synthesis and LC–MS, LC–MS/MS, and NMR analyses of safflomide

Safflomide (*N*-caffeoyltryptamine; Fig. 1) was synthesized using tryptamine and caffeic acid as described previously [12–16]. The synthesis was simple, and the yield of *N*-caffeoyltryptamine was greater than 50%. Synthesized products were purified by HPLC and analyzed by LC–MS and LC–MS/MS as described in the Materials and Methods. LC–MS chromatogram showed a major peak, and the major signal from the peak was obtained at mass/charge (*m/z*) units of 323, which is identical to the *m/z* units of *N*-caffeoyltryptamine (Fig. 2A).

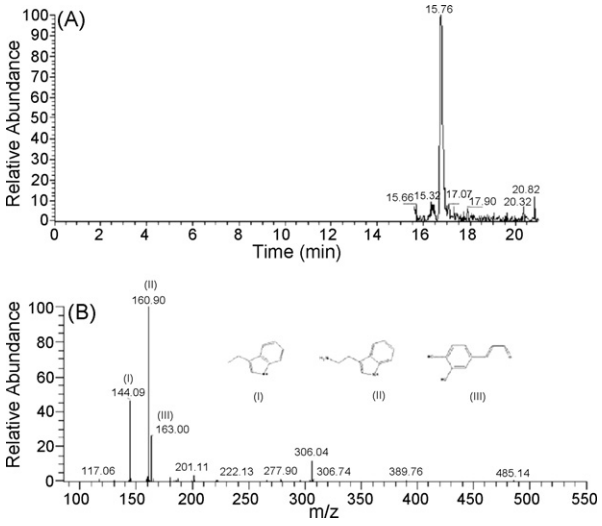

Fig. 2. LC–MS and LC–MS/MS. LC–MS was performed with an Agilent 1100 HPLC equipped with a Supelco C18 (3 μm particle size, 250 mm × 3.0 mm) reversed-phase column operated at 25 °C at a flow rate of 0.25 mL/min. MS/MS was also performed using LCQ Classic ion-trap mass spectrometer. Electrospray ionization (ESI) with MS/MS was performed in positive ion mode to monitor daughter ions from *m*/*z* 85 to 350, with the following conditions: sheath gas flow rate, 70 (arbitrary units); aux gas flow rate, 10 (arbitrary units); spray voltage, 4.50 kV; heated capillary temperature, 220 °C; capillary voltage, −4.0 V; tube lens offset, 25 V. The collision energy for the MS/MS was set at 30%.

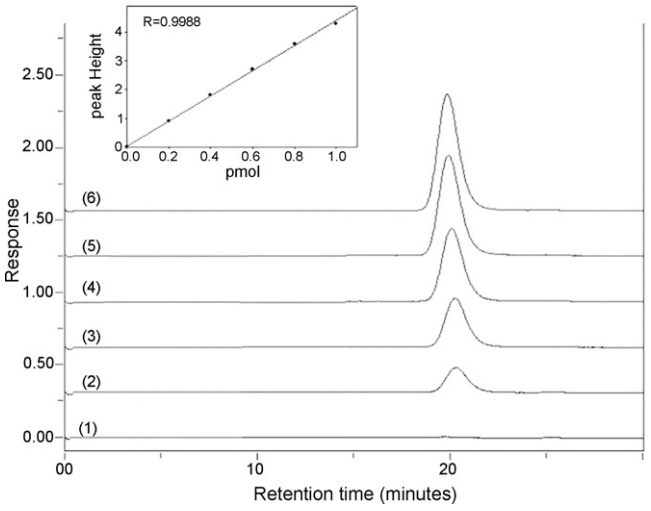

Fig. 3. Chromatograms of safflomide in standard samples. Safflomide standards 1 (0 pmol), 2 (0.2 pmol), 3 (0.4 pmol), 4 (0.6 pmol), 5 (0.8 pmol), and 6 (1 pmol) were injected into a high-performance liquid chromatograph, and chromatograms were collected by using an electrochemical detector poised at 800 mV.

The major peak with the *m*/*z* units of 323 is most likely from the synthesized *N*-caffeoyltryptamine. To confirm the major signal as *N*-caffeoyltryptamine, LC–MS/MS experiments were performed using the product of the major signal in LC–MS. As shown in Fig. 2B, LC–MS/MS spectrum showed three peaks (*m*/*z* = 144, 160, and 163), which were ions of 3-ethylindole (I), tryptamine (II), and caffeic aldehyde (III), respectively. All these three are daughter ions derived from *N*-caffeoyltryptamine, indicating that the compound in the peak of the LC–MS chromatogram is safflomide. The structure of safflomide was also confirmed by NMR analyses: 1H NMR (d_6-DMSO, 400 MHz) δ: 7.38 (1H, d, J = 15.6 Hz, H-7), 7.20 (1H, s, H-13), 7.09 (1H, t, J = 7.3 Hz, H-16), 6.88 (1H, dd, J = 8.2, 1.4 Hz, H-5), 6.41 (1H, d, J = 15.6 Hz, H-8), 7.58 (1H, d, J = 8.2 Hz, H-18), 7.00 (1H, t, J = 7.3 Hz, H-17), 6.80 (1H, d, J = 8.7 Hz, H-4), 7.04 (1H, s, H-1), 7.37 (1H, d, J = 7.8 Hz, H-15), 3.53 (1H, dt, J = 6.0, 6.9 Hz, H-10), 2.94 (1H, t, J = 7.3 Hz, H-11), 8.19 (1H, t, J = 5.5 Hz, H-a), 10.86 (1H, s, H-b); 13C NMR (d_6-DMSO, 100 MHz) δ: 165.7 (C, C-9), 147.5 (C, C-3), 145.7 (C, C-2), 139.3 (C, C-7), 136.4 (C, C-14), 127.4 (C, C-19), 126.6 (C, C-6), 122.8 (C, C-13), 121.1 (C, C-16), 120.6 (C, C-5), 118.8 (C, C-8), 118.4 (C, C-18), 118.5 (C, C-17), 116.0 (C, C-4), 114.0 (C, C-1), 112.0 (C, C-12), 111.5 (C, C-15), 39.8 (C, C-10), 25.5 (C, C-11).

The amide bond in the structure was also confirmed by the following data: two-bond HMBC correlation between H-a and C-9, COSY correlation between H-a and H-10, two-bond HMBC correlation between H-8 and C-9, three-bond HMBC correlation between H-7 and C-9, and three-bond HMBC correlation between H-10 and C-9. Thus, the structure of the synthesized product was determined as being 3-(3,4-dihydroxy-phenyl)-*N*-[2-(1*H*-indol-3-yl)-ethyl]-acrylamide (*N*-caffeoyltryptamine).

3.2. Development of HPLC method

A HPLC method was developed using the synthesized *N*-caffeoyltryptamine as a standard. In this experiment, the synthesized safflomide in standard samples was determined using the HPLC equipped with Nova-Pak C18 column and an electrochemical detector with four electrodes. Several different buffer conditions were tested to improve the resolution of the compound, and an isocratic buffer (50 mM NaH_2PO_4 (pH 4.3), 40% methanol) was selected for optimizing the separation, detection and preparation of samples. The HPLC separation might be improved by adopting different buffer conditions. However, it should be noticed that any variation in buffer conditions might alter retention and total analysis time, and also might cause safflomide to be insoluble, because the compound is fairly hydrophobic. The chromatograms of safflomide standards (0.2, 0.4, 0.6, 0.8, and 1 pmol) are shown in Fig. 3. Total HPLC running time for the assay was less than 30 min, and safflomide was detected at a retention time of 21 min. To optimize applied potentials at the sequential electrodes, hydrodynamic voltammograms of safflomide were generated as described previously [17] (data not shown here). The potential at which half of the safflomide is oxidized is approximately 520 mV, and the potential at which safflomide is fully oxidized is greater than 840 mV. Therefore, all the assays in this study were performed using the electrochemical detector with four electrodes sequentially set at potentials of 100, 300, 550, and 840 mV. Using the detector, a satisfactory linear response for safflomide was also obtained at the concentrations between 0.1 and 10 pmol (correlation coefficient (R) = 0.9988).

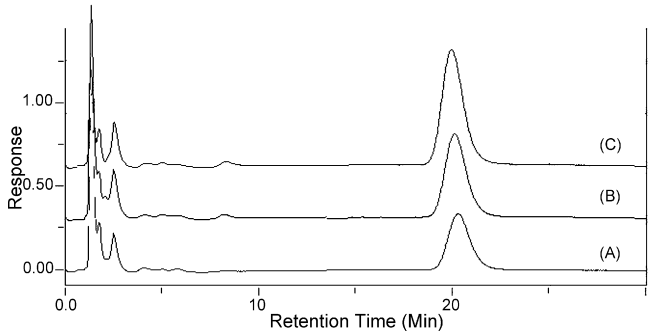

Fig. 4. HPLC assay of safflomide in plasma samples from whole blood collected from Swiss Webster mice. Safflomide was added to the blood samples to give final concentrations of 0.4 μM (A), 0.6 μM (B), and 0.8 μM (C). Chromatograms were collected by using an electrochemical detector poised at 800 mV.

3.3. HPLC measurement of safflomide in plasma samples

Although all the assay conditions were optimized to separate safflomide in standard samples, the method still needed to be validated in biological samples. Therefore, the HPLC assay was further tested using plasma samples. For preparing plasma samples, whole blood was collected from Swiss Webster mice, and safflomide was added to the blood samples to provide final concentrations of 0.4, 0.6, and 0.8 μM. The blood samples were extracted and the HPLC samples prepared and assayed as described in Materials and Methods. The chromatograms of safflomide in plasma samples are shown in Fig. 4. As expected, safflomide was detected at the same retention time of 21 min as standard samples, suggesting that the developed HPLC method can be applicable to standard as well as plasma samples. Also, the extraction recovery from the plasma samples was determined, because the extraction method was employed in preparing plasma samples. First, safflomide was added to the plasma (10 pmol/μL), and the samples were extracted twice as described in Materials and Methods. The amount of safflomide in the extracted samples was quantified by the HPLC method and compared to the amount originally added to the plasma sample. The first and second extractions were performed and analyzed, and the recovery was determined using three different samples ($n = 5$). In five replicate preparations, the recovery of safflomide in the first and second extractions was more than 97% and less than 3%, respectively, and the recovery was very consistent (Table 1). These data indicate that the first extraction may be enough for preparing cell samples, because the recovery of safflomide in the plasma samples was more than 97% in the replicate preparations. As demonstrated above, the HPLC method with the extraction procedure can provide excellent precision for determining safflomide in plasma samples.

Table 1
The extraction recovery (pmol/μL) of safflomide from the plasma samples

	First extraction	Second extraction
Safflomide	9.7 ± 0.3	0.3 ± 0.1

Safflomide (10 pmol/μL) was added to plasma samples ($n = 5$), and extracted twice as described in Material and Methods.

3.4. Determination of safflomide in mouse plasma following oral administration

Safflomide has been reported to contain several biological activities, and the activities are likely to be dependent on its concentrations [10–12]. Bioavailability is the physiological availability of a compound at a given amount, and is dependent mainly on initial administrated amount, absorption, metabolism, tissue distribution and excretion. Studies are often performed using several different doses and different routes to determine bioavailability of an administrated compound. Therefore, in this study, plasma concentrations of safflomide following two oral administrations (1 and 3 mg/30 g body weight) were measured as a way to determine its concentrations after the oral administrations. In this experiment, the HPLC method developed in this study was used to measure *N*-caffeoyltryptamine in plasma samples prepared from the oral dosing groups. Also, the peak at retention time of 21 min in collected plasma samples was validated as safflomide by LC–MS and LC–MS/MS spectroscopic methods (data not shown here). The average concentrations of safflomide ($n = 5$) in the plasma collected at 0, 5, 10, 15, 20, 25, 30, and 35 min were 0, 0, 0, 15, 50, 10, 0, and 0 μM after the first (1 mg/30 g body weight) oral administration and 0, 0, 0, 4, 14, 3, 0, and 0 μM after second (3 mg/30 g body weight) oral administration. As shown in Fig. 5, a fair amount of safflomide could be detected around 10 min after the oral administration (3 mg/30 g body weight), and the highest amount was detected at around 15 min. After 15 min, plasma concentrations began to decrease rapidly, suggesting that safflomide taken orally may be metabolized quickly after 15 min. The oral administration (1 mg/30 g body weight) also produced similar patterns; the detection beginning around 10 min, highest at around 15 min, and decreasing after 15 min. It is noticeable that the areas under the curves of the plasma concentrations of safflomide over time are proportional to the doses orally administrated to mice, suggesting that plasma concentrations may be used for calculating oral administration

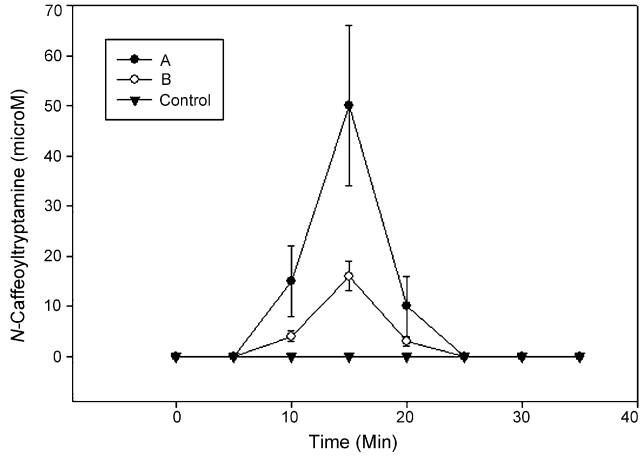

Fig. 5. Determination of safflomide in mice plasma following oral administration to Swiss Webster mice. Safflomide 3 mg/30 g body weight (A) and 1 mg/30 g body weight (B) was orally administrated to the mice. The concentrations of *N*-caffeoyltryptamine in plasma samples were determined as described in Material and Methods.

doses. For instance, based on Fig. 5, the range of plasma concentrations of safflomide are expected to be around 5–16 μM after the oral administration (1 mg/30 g body weight). Indeed, the plasma concentrations were approximately 4–14 μM in the plasma samples collected at 10, 15, and 20 min after the oral administration of 1 mg/30 g body weight.

In this study, safflomide was chemically synthesized and HPLC method was developed to measure the safflomide in plasma samples. To our knowledge, this is the first report regarding the chemical synthesis of safflomide and HPLC method able to quantify safflomide in plasma samples with excellent detection limit and consistent reproducibility. This method may be useful in future studies for investigating cellular and molecular functions, absorption and metabolism of safflomide and its analogues.

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