Theroleofchemicalfingerprinting: applicationto Ephedra

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Abstract

Ephedra sinica, known as Ma Huang, is one of the oldest medicinal herbs in Traditional Chinese Medicine (TCM). Preparations, namely teas, of E. sinica have been used for over 5000 years as a stimulant and as an antiasthmatic. In the West, extracts of E. sinica, E. intermedia or E. equisetina are most commonly used in dietary supplements as a stimulant and to promote weight loss. More than 50 species of Ephedra are native to both hemispheres, but the detection of ephedrine alkaloids has been limited to species in Eurasia. Currently, methods exist to quantitate the ephedrine alkaloids in extracts of plant material or dietary supplements, but the methods are not able to verify the extract is of an Ephedra species. Reverse phase high performance liquid chromatography with photodiode array detection was applied for the chemical fingerprinting of the Ephedra species. Two regions of comparison were determined in the chromatograms at 320 nm. The series of peaks between 52 and 64 min confirms an Ephedra species is being analyzed. The aforementioned peaks also could distinguish between Ephedra species from Eurasia, North America and South America. Peaks at ca. 57 and 59 min were isolated and determined to be two new compounds, 4-(2-eicosyloxycarbonyl-vinyl)-benzoic acid and 4-(2-docosyloxycarbonyl-vinyl)-benzoic acid respectively. Authentication of ground plant material as Ephedra can be achieved by this chemical fingerprinting method.

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1. Introduction

During the mid 1990s, the botanical dietary supplement market grew exponentially. This increased demand for herbal products was met by a flood of new companies wanting their share of this billion dollar industry. Unfortunately, the scientific support behind these products has not been able to keep up with the industries rapid expansion (Cardellina, 2002). The lack of science has created an industry where quality is sometimes compromised. One common method used by the industry for quality control is analyzing the product for the presence of chemical markers known to be present in the specific herbal they happen to be marketing, whether the markers are the cause of the physiological affect or not. Even though this has been the acceptable method for quality control, the presence of the chemical markers do not always guarantee an individual is getting the actual herbal stated by the product label, especially if the product has been spiked with the chemical markers. The quantitation method for the chemical markers will confirm the compounds presence, but it may not confirm the presence of the plant material known to contain the chemical markers. Authentication of the plant material may be possible by a chemical fingerprint of the botanical. Chemical fingerprinting is an additional method that must be included as a quality control method in order to confirm or deny the plant material being used for the manufacturing of a product. Products claiming to contain Ma Huang are a prime example of a botanical where chemical fingerprinting is a must for authentication purposes.

Ephedra sinica, known as Ma Huang, is one of the oldest medicinal herbs in traditional Chinese medicine (TCM). Preparations, namely teas, of E. sinica have been used for over 5000 years as a stimulant and as an antiasthmatic (Bensky and Gamble, 1993; Chen and Schmidt, 1926). The genus of Ephedra, which contains
over 50 species, belongs to the family Ephedraceae (Caveney et al., 2001). The shrubs, which reach approximately one meter in height, grow in semiarid and desert conditions in both hemispheres across six continents (Price, 1996).

In the West, dietary supplements containing Ma Huang extracts have become one of the top selling weight loss and endurance enhancing products on the market. Currently, it is thought that the number of people consuming Ephedra is in the millions. However, this growth has not been without its controversy, over the last decade state and federal governments have regulated Ephedra containing products due to a growing number of reported adverse events caused by misuse or abuse of the herb (Food Drug Administration, 1997; Department of Health and Human Services, 2000; Haller and Benowitz, 2000; Zaacks et al., 1999). These adverse events are thought to be due to a series of optically active alkaloids.

Six optically active alkaloids, the ephedrine alkaloids (Fig. 1), are considered the active constituents in Ma Huang. The concentration of ephedrine alkaloids can vary from 0.02 to 3.40% in the aerial parts of the plant (Leung and Foster, 1996). (−)-Ephedrine (1) is the major isomer. The minor ephedrine alkaloids include (+)-pseudoephedrine (2), (−)-methylephedrine (3), (+)-methylpseudoephedrine (4), (−)-norpseudoephedrine (5) and (+)-norpseudoephedrine (6). The pharmacological studies have shown 1 to be a sympathomimetic agonist at both the α- and β-adrenergic receptors, which leads to an increased cardiac rate and contractility, to peripheral vasoconstriction, to bronchodilation, and to central nervous system (CNS) stimulation (Fouad-Tarazi et al., 1995; Walker et al., 1998). Ephedrine (1) is not the only alkaloid used in products, over the counter decongestant preparations commonly contain 2. Weight loss and enhanced performance in endurance training and body building may be due to the CNS stimulation and thermogenic properties of 1 (Walker et al., 1998). Due to the ephedrine alkaloid activities indicated in previous studies, contraindications are given for individuals with hypertension or other cardiovascular diseases, glaucoma, diabetes and hyperthyroidism (Fetrow and Avila, 1999; Tyler, 1999).

Although E. sinica has been the primary source for ephedrine alkaloids, other species of Ephedra throughout Eurasia contain the active constituents: E. equisetina,

![Fig. 1. The structures of the ephedrine alkaloids and two new compounds isolated from E. sinica.](image1)

![Fig. 2. The HPLC chromatograms obtained by an alkaloid method: an extract of E. sinica (A), an extract of E. aspera (B), an extract of a dietary supplement only containing E. sinica (C), an extract of a dietary supplement containing E. sinica as part of a blend of herbals (D) and an extract of G. biloba spiked with ephedrine alkaloids (E).](image2)
E. intermedia, E. gerardiana, E. alata, E. distachya, E. botschantzevii, E. fragilis, E. major, E. minuta, E. monosperma, E. pachyclada, E. likiangensis, E. saxatilis, E. lomatolepis, E. lepidosperma, E. przewalskii and E. regeliana (Caveney et al., 2001). Although three articles have been published which claim the presence of ephedrine alkaloids in some North and South American species of Ephedra, the results remain unconfirmed (Caveney et al., 2001; Willaman and Schubert, 1964; Wink, 1998). Currently, the Ephedra species of the Americas are considered devoid of the ephedrine alkaloids (Caveney et al., 2001).

Due to the number of people consuming Ephedra and the number of adverse events reported to the Food and Drug Administration (FDA), continued research is needed to ensure quality of the products being sold. Several factors may have contributed to the adverse effects. These include consumer misuse, manufacturer abuse and contraindication, hypersensitivity and/or drug interaction. Of the three, manufacturer abuse deals with the spiking of Ephedra plant material and/or products with synthetic stimulants or the synthetic ephedrine alkaloids (Ross et al., 1999). Methamphetamine (MDMA) is easily synthesized from ephedrine, and may be one of the spiking adulterants present in a product (Skinner, 1990).

At this time a number of methods have been reported for the quantitation of the ephedrine alkaloids present in some Ephedra species: chiral gas chromatography (Betz et al., 1997), capillary electrophoresis (Chinaka et al., 2000), high performance liquid chromatography with UV detection (Gurley et al., 1998) and liquid chromatography with mass spectrometry detection (Gay et al., 2001). Although important, these methods are each limited in their ability to ensure identity of the plant material being used in the product. Thus, an HPLC method is here in reported where a chemical fingerprint was developed for a number of Ephedra species, some known to contain ephedrine alkaloids and some known to be ephedrine alkaloid free. The creation of the chemical fingerprint also led to the isolation of two new compounds, 7 and 8.

2. Results and discussion

For illustrative purposes, five different samples were analyzed with an in house HPLC ephedrine alkaloid quantitative method. The five samples were E. sinica ground plant material (A), E. aspera ground plant material (B), a dietary supplement claiming to contain only E. sinica (C), a dietary supplement containing E. sinica as part of a herbal blend (D) and a dietary supplement containing Ginkgo biloba spiked by us with 1 (E). As shown in Fig. 2, it is difficult to detect which of the products contains Ginkgo spiked with 1. The peak representing 1 is marked in each chromatogram. On the
same note, most would not realize chromatogram B was indeed an Ephedra species due to the lack of ephedrine (I) detection.

Although the quantitation method is useful for the standardization of the ephedrine alkaloids to ensure quality in a product by making sure the ephedrine alkaloids are present and not in quantities above the maximum daily dose, the method falls short of Ephedra plant authentication. Many companies purchase bulk ground plant material from an outside source, but do not have a method for the authentication of the material or do not receive authentication information from the supplier. Although they will analyze the material for ephedrine alkaloid content, they may not have a method for Ma Huang confirmation. Quality concerns can be overcome by simple testing of the starting plant material. It can determine if the plant is in fact Ephedra, which then can be used to determine if another plant species had been spiked with ephedrine alkaloids. At the same time a plant authentication method can distinguish between species within the genus. The chemical fingerprint method developed in this study was able to verify an Ephedra species present in ground plant material and it was able to distinguish between the Ephedra species grown in North America, South America and Eurasia.

Initial fingerprint development began by extracting authenticated E. sinica with ethanol. The extract was then analyzed on a series of stationary phases with a gradient solvent system (95 water/5 acetonitrile to 100 acetonitrile) over 1 h. Three UV wavelengths were analyzed: 210, 254 and 320 nm. Regardless of the stationary phase, a level baseline could not be achieved. This was also the case after changing the mobile phase gradient and percentages. Since a level baseline was not achieved with ethanol, the extraction solvent was changed to acetone. Due to the decrease of co-extractives with acetone, an optimal system for HPLC was achieved. The acetone extract was analyzed on a Waters X Terra RP 18 column. A level baseline was achieved. An optimal mobile phase gradient was then determined for the fingerprint.

Fig. 4. HPLC comparison of chromatograms at 320 nm of E. sinica (A), E. gerardiana (B), E. nevadensis (C), E. fominea (D), E. distachya ssp. helvetica (E) and G. biloba spiked with ephedrine alkaloids (F).
comparison of the chromatograms at 210, 254 and 320 nm found the absorbance at 320 nm to be the most advantageous (Fig. 3).

Once the fingerprint method had been developed through the use of authenticated plant material, other species of Ephedra had to be tested to ensure its usefulness. All species of Ephedra tested are listed in the experimental section. A comparison of the fingerprints of a number of the Ephedra species obtained is shown in Fig. 4. The first five chromatograms are of Ephedra species, while the sixth chromatogram is of an ephedrine alkaloid spiked G. biloba extract. A series of peaks between 52 and 64 min are of interest. The UV spectrum for peaks 7 and 8 are the same and shown in Fig. 5. All Ephedra species analyzed contained this series of peaks between 52 and 64 min with slight variation between the species in the Americas and Eurasia. More importantly, those peaks present at 320 nm in the Ephedra species were not present in the ephedrine alkaloid spiked G. biloba extract (Fig. 4). The region was also absent in the acetone extract of Ginseng (data not shown). This shows the fingerprint is capable of determining if ground plant material is from an Ephedra species. The North American and South American species of Ephedra could also be distinguished from the Eurasian species of Ephedra (Fig. 6). The differences are marked in Fig. 6.

The fingerprint method was validated by testing a number of populations within a single species of Ephedra. Fig. 7 compares the chromatograms obtained from four different populations of E. trifurca. The key regions are present in all four chromatograms, and the chromatograms all have the same relative features. E. trifurca was collected in populations from Texas and New Mexico.

The development of the fingerprint method led to the isolation of two new compounds, 7 and 8 from E. sinica. Both compounds were isolated by high performance flash chromatography and prep HPLC. The representative peaks are shown in Fig. 6 and the structures in Fig. 1.
3. Conclusions

The availability of analytical methods for the analysis of ground plant material is paramount in ensuring quality in dietary supplement and herbal products. It is possible a manufacturer will purchase in bulk, ground plant material, but will only have the word of the supplier for sample authentication. As a powder, visual authentication of the plant is not possible. With this fingerprint method, manufacturers as well as consumer groups can test the ground plant material to confirm or deny the presence of an Ephedra species. This will help stop possible spiking of plant material other than Ephedra with synthetic ephedrine as well as spiking of a species of Ephedra known to be devoid of the ephedrine alkaloids. Chemical fingerprinting is only one step towards ensuring quality in dietary supplements.

4. Experimental

4.1. Plant material

*E. antisyphilitica*, *E. aspera*, *E. californica*, *E. foeminea*, *E. coryi*, *E. cutleri*, *E. equisetina*, *E. fasciculata*, *E. nevadensis*, *E. pendunculata*, *E. torreyana ssp. powelliorum*, *E. torreyana*, *E. trifurca*, *E. viridis*, *E. aspera × E. trifurca*, *E. distachya*, *E. distachya ssp. helvetica*, *E. equisetina*, *E. gerardiana*, *E. intermedia*, *E. major*, *E. ochreata*, *E. sinica* and *E. triandra* were studied and voucher specimens are stored at The University of Mississippi Herbarium (MISS).

4.2. Solvents

Acetonitrile, acetone, absolute ethanol and methanol were purchased from Fisher Scientific (Fair Lawn, NJ).
and nanopure water was prepared by filtering distilled water through a 45 μm filter.

4.3. Sample preparation

Approximately 500 mg of ground plant material was placed in a Falcon Blue Max Jr. 15 ml polystyrene conical tube (Becton Dickinson Labware, Franklin Lakes, NJ) with 6.0 ml of acetone and sonicated 15 min in a Fisher Scientific Ultrasonic Cleaner (Pittsburgh, PA). After sonication, the sample was centrifuged for 10 min in a Centrifuge (Precision Scientific, Chicago, IL). The supernatant was transferred by pipette to a borosilicate 6 Dram sample vial (VWR, South Plainfield, NJ). The extraction was repeated two more times and the respective supernatants were combined and the acetone removed (Savant Speed Vac Plus SC210A with a Savant Refrigerated Vapor Trap, Holbrook, NY). To the dried extract were added 5.0 ml of absolute ethanol. Once the extract had dissolved, 2.0 ml were filtered (the first 0.5 ml were discarded) through a 45 μm Nylon filter (Phenomenex, Torrance, CA) into an HPLC borosilicate sample vial (VWR, South Plainfield, NJ) for analysis.

4.4. HPLC fingerprinting

HPLC/PDA analysis was performed on a Waters Alliance 2695 Separations Module with a Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). The column was a Waters X Terra RP18 5 μm (150 × 4.6 mm, 5 μm particle size) (Waters, Milford, MA, USA). The mobile phase consisted of nanopure water (A) and acetonitrile (B). After the column was equilibrated to 40 °C, the gradient mobile phase began by holding at 75A/25B for 10 min. Then the system mixture changed to 0A/100B over 45 min and was held at 100B for 10 min. Total run time was 65 min. The injection volume was 10 μl and the flow rate was 1.00 ml/min. Sample analysis was processed by Waters Millennium32 software (Waters, Milford, MA, USA).

4.5. Isolation method

Plant material (E. sinica; 450 g) was extracted with acetone (1 l) by sonication for 30 min and filtered. The extraction procedure was carried out a total of four times and the filtrates were combined. The filtrate was concentrated to dryness in vacuo (14.1 g). An aliquot of the extract (1.50 g) was applied to Horizon Biotage Inc. (Biotage, Inc., Charlotteville, VA, USA), HPFC system (High Performance Flash Chromatography) using normal phase silica gel (Si 40M; Flow rate: 15 ml/min), eluted with n-hexane (200 ml), n-hexane–EtOAc mixtures (90:10; 80:20; 70:30; 60:40; 50:50; each 200 ml), n-hexane–EtOAc–MeOH mixtures (50:50:5; 50:50:10; 50:50:20; each 200 ml), and MeOH (0.5 l). According to HPLC profiles, the fractions 1–20 were pooled together and subjected to Prep HPLC. The new compounds 7 and 8 were isolated by preparative HPLC on a Waters Alliance 2695 Separations Module with a Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). The column was a Waters X Terra RP18 10 μm (250 × 10 mm, 10 μm particle size) (Waters, Milford, MA, USA). The isocratic mobile phase consisted of nanopure water (A), acetonitrile (B) and methanol (C) [5:85:10] at 3.0 ml/min. After the column temperature reached 40 °C, 100 μl were injected and detection took place at 320 nm. Compounds 7 and 8 were collected separately. The mobile phase in each fraction was removed to give a white solid (2.0 and 1.5 mg respectively) for 1H and 13C NMR analysis.

4.6. Fingerprint validation

The extraction method listed above used a polypropylene tube as the extraction vessel. Five points can be made to confirm polymer contamination was not responsible for the absorbances between 52 and 64 min. First, species from Eurasia, North America and South America had different profiles in the same region. Second, in both the ginkgo and ginseng extracts the peaks were not detected. At the same time the large scale isolation of the new compounds took place in the absence of polypropylene. Also, the small scale extraction was carried out in a glass vessel with acetone and a blank extraction with acetone was carried out in the polypropylene tube. In all three cases, the peaks shown in the Ephedra fingerprint were not detected.

4.7. Structure elucidation data

4.7.1. Compound 7

4-(2-Eicosyloxyvinyl)-benzoic acid; Negative HRESIMS; m/z 471.3900 [M–H]– (calc. for C30H48O4 472.3553); 1H NMR data (500 MHz, in C5D5N); 7.90 (1H, d, \( J = 15.8 \) Hz, H-β), 7.61 (2H, d, \( J = 7.5 \) Hz, H-2 and H-6), 7.13 (2H, d, \( J = 7.5 \) Hz, H-3 and H-5), 6.60 (1H, d, \( J = 15.8 \) Hz, H-α), 4.25 (2H, t, \( J = 6.5 \) Hz, H-1'), 1.65 (2H, p, \( J = 6.8 \), 13.9, 14.1 Hz, H-2'), 1.23-1.25 \([\text{[CH}_2\text{]}_2\]), 0.82 (3H, t, \( J = 6.0 \) Hz, H-20').

4.7.2. Compound 8

4-(2-Docosyloxyvinyl)-benzoic acid; Negative HRESIMS; m/z 499.4210 [M–H]– (calc. for C32H52O4 500.3866); 1H NMR data (500 MHz, in C5D5N+DMSO-δ6); 8.99 (1H, brs, COOH), 7.59 (1H, \( J = 15.9 \) Hz, H-β), 7.53 (2H, d, \( J = 8.4 \) Hz, H-2 and H-6), 6.87 (2H, d, \( J = 8.4 \) Hz, H-3 and H-5), 6.36 (1H, \( J = 15.9 \) Hz, H-α), 4.14 (2H, t, \( J = 6.6 \) Hz, H-1'), 1.67 (2H, p, \( J = 6.8 \), 13.9, 14.1 Hz, H-2'), 1.28–1.30 \([\text{[CH}_2\text{]}_2\]);
H$_2$-3$\rightarrow$H$_2$-21'), and 0.88 (3H, $t$, $J=6.0$ Hz, H-22'); $^{13}$C-NMR data (125 MHz, in C$_5$D$_5$N and DMSO-$d_6$): δ?167.2 (s, COO), 162.0 (s, COOH), 145.1 (d, C-β), 130.5 (x3) (s, C-1; d, C-3 and C-5), 125.9 (s, C-4), 116.4 ($\times$2) (d, C-2 and C-6), 114.8 (d, C-α), 64.2 ($t$, C-1$'$), 32.1 ($t$, C-21$'$), 29.1-30.2 ($t$, CH$_2$)$_n$; C-3$'$→C-19$'$, 26.2 ($t$, C-2$'$), 22.8 ($t$, C-20$'$), 14.1 (q, C-22$'$).

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