The Rapid Identification of Isoflavonoids from *Belamcanda chinensis* by LC-NMR and LC-MS

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The use of hyphenated LC-NMR and LC-MS techniques for the purpose of directly identifying the major constituents of *Belamcanda chinensis* was investigated. Reversed-phase isocratic chromatography was performed using an acetonitrile–water solvent system on a C18 column. The NMR spectrum yielded five main peaks, whose analysis revealed them to be 5, 6, 7, 3′-tetrahydroxy-4′-methoxyisoflavone (1), tectorigenin (2), iristectorigenin A (3), irigenin (4), and irisflorentine (5). The identification of these constituents was confirmed by performing LC-ESI-MS experiment. This study shows that hyphenated LC-NMR and LC-MS can be used for the rapid (70 min) identification of the isoflavonoids.

Key words LC hyphenated technique; LC-NMR; LC-MS; isoflavonoid; *Belamcanda chinensis*

The hyphenated techniques of LC-NMR and LC-MS are increasingly used for phytochemical analysis. These techniques combine the separation power of HPLC with the structural information provided by NMR and MS. The major advantages over the traditional off-line techniques are rapidity and efficiency.[1] The traditional approach required various chromatographic techniques, including HPLC, followed by MS and NMR experiments.[2] This strategy is time-consuming and labor-intensive.

Efficient detection and rapid characterization of analytes is the one of the most common challenges encountered in the study of complex biological matrices, such as crude plant extracts.[3] However, the development of novel techniques, such as hyphenation of HPLC, NMR and MS, offer the potential of rapid target component identification without the need for extensive purification.[4]

Belamcanda chinensis (L.) DC. (Iridaceae), a perennial shrub that grows on hillsides in East Asia, including the Korean peninsula, has been used as Chinese traditional medicine for the treatment of throat ailments, such as asthma and tonsillitis.[5] A number of studies concerning the constituents of *B. chinensis* have been previously reported.[6—9] However, prior separation and purification of the constituents was necessary for NMR detection. The aim of this study is to identify the major constituents of *B. chinensis* more rapidly and efficiently. We report the application of the combined use of LC-NMR and LC-MS for the separation and structure elucidation of the five isoflavonoids present in *B. chinensis*.

Results and Discussion

Figure 1 shows the LC chromatogram at 280 nm of an ethanol extract from the root of *B. chinensis*, which was extracted from the LC-NMR data (Fig. 2). The LC/UV trace revealed 5 major peaks (t_R=11.5, 22.7, 24.8, 26.9, 55.8). The separation was performed within 70 min using an optimized isocratic acetonitrile-D_2O solvent system. The isocratic solvent system was more favorable than gradient condition because in the isocratic condition, the solvent composition of the NMR flow cell was kept constant, we could acquire more stable NMR spectra.

Minor peaks were not considered in this analysis because chromatographic resolution was insufficient and/or the signal-to-noise (S/N) ratio was unsatisfactory. The resulting contour plot is shown in Fig. 2. The *x* axis corresponds to ^{1}H-NMR chemical shift values while the *y* axis reflects the chromatic retention time. The elution profile of the five main compounds can be easily seen. For the sake of clarity, ^{1}H-NMR spectra were extracted at the corresponding peak maxima, which are shown in Fig. 3. Additional evidence for the identity of the isoflavonoids was obtained from LC-ESI-MS experiments performed in the positive mode.

The UV spectrum of compound **1** showed maxima at 264 and 325 nm, which suggested the presence of an isoflavone

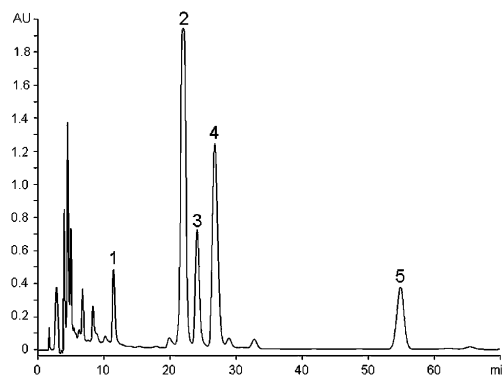

Fig. 1. LC Chromatogram of the Ethanol Extract of the Root from *B. chinensis* with Detection at 280 nm (Extracted from the LC-NMR Experiment)

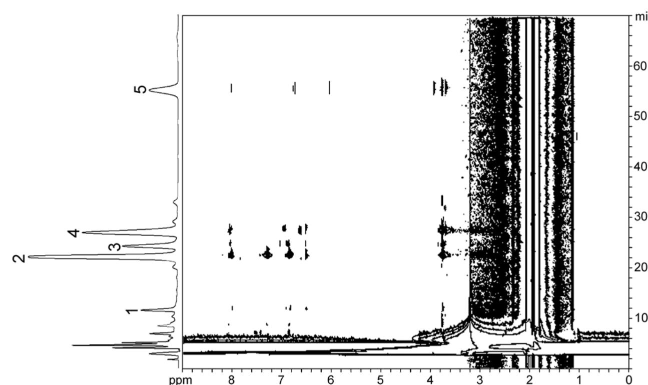

Fig. 2. Contour Plot of the LC-NMR Coupling

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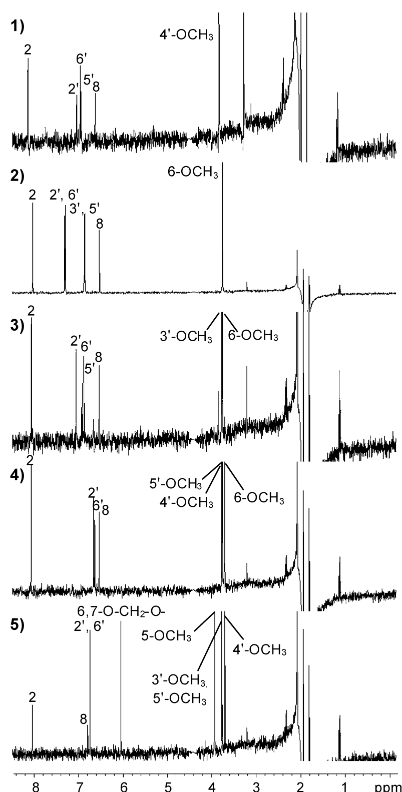
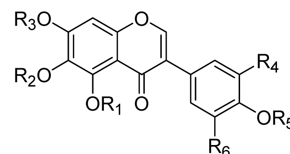


Fig. 3. On-flow ^{1}H-NMR Spectra of Each Peak



	R_1	R_2	R_3	R_4	R_5	R_6
1	H	H	H	OH	CH_3	H
2	H	CH_3	H	H	H	H
3	H	CH_3	H	OCH_3	H	H
4	H	CH_3	H	OCH_3	CH3	OH
5	CH_3	-CH_2-		OCH_3	CH_3	OCH_3

Fig. 4. Structures of the Isoflavonoids of *B. chinensis* Identified by LC-NMR and LC-MS Analyses

skeleton.[10,11] The ^{1}H-NMR spectrum of compound **1** showed three singlets (δ 8.00, 6.51, 3.74), one *meta*-coupled doublet (J=2 Hz) (δ 6.92), one *ortho*, *meta*-coupled double doublet (J=2, 8 Hz) (δ 6.83) and one *ortho*-coupled doublet (J=8 Hz) (δ 6.82). Two protons appearing at δ 8.00 and δ 6.51 could be assigned to H-2 and H-8, respectively. The signal for three protons at δ 3.74 indicated the presence of a methoxyl group and was assigned to OCH_3-4′. The doublets at δ 6.92, δ 6.83 and 6.82 were suggestive of H-2′, H-6′ and H-5′, respectively. LC-ESI-MS yielded a pseudo-molecular ion peak at m/z 317 $[M+H]^+$. Based on these spectral data, compound **1** was assumed to be 5,6,7,3′-tetrahydroxy-4′-methoxyisoflavone. The interpretations of these spectral data were largely consistent with those of Ito *et al.*[7] Therefore, compound **1** was characterized as 5,6,7,3′-tetrahydroxy-4′-methoxyisoflavone.

Compound **2** exhibited a $[M+H]^+$ ion peak at m/z 301. The presence of an isoflavone skeleton was suggested by the UV spectrum (λ_{max} 265, 330). Compound **2** showed similar ^{1}H-NMR spectrum to compound **1**. The double doublets at δ 7.28 and 6.84 were assigned to H-2′, 6′ and H-3′, 5′, respectively. A methoxyl group at δ 3.74 was suggestive of OCH_3-6. Thus, compound **2** was assumed to be tectorigenin.

The ^{1}H-NMR spectrum of compound **3** was very similar to that of compound **1**, except for the presence of two methoxyl groups (OCH_3-3′ and OCH_3-6) at δ 3.76 and 3.75, respectively. Compound **3** was assumed to be iristectorigenin A.

The spectrum of compound **4** indicated the presence of three methoxyl groups (OCH_3-5′, OCH_3-4′, OCH_3-6) at δ 3.75, 3.74 and 3.69, respectively. The H-5′ in compound **3** was replaced by OCH_3-5′ in compound **4**. Thus, compound **4** was characterized as irigenin.

In the ^{1}H-NMR spectrum of compound **5**, H-8 was found at δ 6.77, 6,7-O–CH_2–O– at δ 6.03 and four methoxyl groups (OCH_3-5, OCH_3-3′, OCH_3-5′, and OCH_3-4′) at δ 3.92, 3.76, 3.76 and 3.69, respectively. LC-ESI-MS yielded a pseudomolecular ion peak at m/z 387 $[M+H]^+$. Compound **5** was deduced to be irisflorentine. The structures are shown in Fig. 4.

In the present study, the major constituents of *B. chinensis* were identified through the combined use of LC-NMR and LC-MS analysis. This technique was more rapid and efficient than traditional methods and the results demonstrate that these hyphenated techniques are readily applicable for the identification of the isoflavonoids of *B. chinensis*.

Experimental

Plant Material and Sample Separation The dried roots of *B. chinensis* were purchased from the Deok-hyun herb shop, Gyundong Market, Seoul. The voucher specimen (BC-001) was stored at the Natural Products Research Center, KIST Gangneung Institute, Gangneung, Korea. Sonication extraction was carried out in an ultrasonic cleaning bath (model RK 158s, Bandelin, Germany). Ten grams of the dried roots of *B. chinensis* was extracted twice with 50 ml ethanol at room temperature for 1 h and filtered through a Whatman No. 1 filter paper. The combined filtrate was concentrated to dryness by rotary evaporation at 40 °C to yield an ethanol extract (200 mg).

Liquid Chromatography-Nuclear Magnetic Resonance Spectroscopy Analysis was performed under ambient conditions using a Prostar 230 ternary gradient pump, a Prostar 430 autosampler (Varian, Walnut Creek, CA, U.S.A.) and a Prostar 335 photodiode array detector (Varian, Mulgrave, Victoria, Australia). Separation was carried out on a 4.6×150 mm Zorbax Eclipse XDB-C18 column (Agilient, Santa Rosa, CA, U.S.A.) with a particle size of 5 μm. Elution was isocratic using a mobile phase consisting of 30% acetonitrile (HPLC grade, Fisher Scientific, Pittsburgh, PA, U.S.A.) and 70% D_2O (Cambridge Isotope Laboratories, Andover, MA, U.S.A.) for 70 min. The flow-rate was maintained at 0.5 ml/min. PDA was performed in the 200—800 nm range and the chromatographic profile was recorded at 280 nm. Fifty milligrams of the *B. chinensis* ethanolic extract was dissolved in 1 ml of methanol and filtered through a 0.45 μm membrane filter. Thirty microliters of this solution was injected into the LC-NMR system.

LC-NMR data were acquired using a Varian NMR system 500 MHz (Varian, Palo Alto, CA, U.S.A.). The system was equipped with an LC-NMR cold flow-probe with a 60 μl flow cell (active volume). ^{1}H-NMR spectra were obtained in on-flow mode. Varian WET solvent suppression was used to suppress the acetonitrile and the residual water peaks. The WET technique used a series of variable tip-angle solvent-selective radio frequency (RF) pulse. Each selective RF pulse was followed by a dephasing field gradient pulse. Free induction decays were collected with 16 K data points, a spectral width of 12000 Hz, a 7.1-μs 90° pulse, a 1.8 s acquisition time, and a 1 s pulse delay. A total of 4 transients were acquired. An exponential

apodization of the FID with line broadening of 1.0 Hz was performed before Fourier transformation. The data were analyzed using Vnmrj software (Varian).

Liquid Chromatography-Mass Spectrometry LC-MS experiments were performed using a Agilent 1100 Series HPLC System interfaced with a 6300 Series Ion Trap LC/MS System (Agilent Technologies, Waldbronn, Germany). Chromatographic conditions were similar to those previously described, except that H_2O (HPLC grade, Fisher Scientific, Pittsburgh, PA, U.S.A.) was used instead of D_2O.

Mass spectrometer conditions are as follows; positive ion mode; mass range, *m*/*z* 100—1000; the capillary voltage, 3500 V; nebulizing gas pressure (N_2), 30 psi; drying gas (N_2) flow rate, 13.0 $l\,min^{-1}$; drying temperature, 350 °C. ChemStation (Agilent Technologies) software was used to operate this LC-MS system.

5,6,7,3′-Tetrahydroxy-4′-methoxyisoflavone (**1**): ESI-MS *m*/*z*: 317 $[M+H]^+$. UV λ_{max} (30% acetonitrile and 70% D_2O): 264, 325 (sh). ^{1}H-NMR (30% acetonitrile and 70% D_2O) δ: 8.00 (1H, s, H-2), 6.92 (1H, d, J=2 Hz, H-2′), 6.83 (1H, dd, J=2, 8 Hz, H-6′), 6.82 (1H, d, J=8 Hz, H-5′), 6.51 (1H, s, H-8), 3.74 (3H, s, OCH_3-4′).

Tectorigenin (**2**): ESI-MS *m*/*z*: 301 $[M+H]^+$. UV λ_{max} (30% acetonitrile and 70% D_2O): 265, 330 (sh). ^{1}H-NMR (30% acetonitrile and 70% D_2O) δ: 8.01 (1H, s, H-2), 7.28 (2H, dd, J=2, 9 Hz, H-2′, 6′), 6.84 (2H, dd, J=2, 9 Hz, H-3′, 5′), 6.50 (1H, s, H-8), 3.74 (3H, s, OCH_3-6).

Iristectorigenin A (**3**): ESI-MS *m*/*z*: 331 $[M+H]^+$. UV λ_{max} (30% acetonitrile and 70% D_2O): 268, 333 (sh). ^{1}H-NMR (30% acetonitrile and 70% D_2O) δ: 8.04 (1H, s, H-2), 7.03 (1H, d, J=2 Hz, H-2′), 6.89 (1H, dd, J=2, 8 Hz, H-6′), 6.58 (1H, d, J=8 Hz, H-5′), 6.51 (1H, s, H-8), 3.76 (3H, s, OCH_3-3′), 3.75 (3H, s, OCH_3-6).

Irigenin (**4**): ESI-MS *m*/*z*: 361 $[M+H]^+$. UV λ_{max} (30% acetonitrile and 70% D_2O): 265, 335 (sh). ^{1}H-NMR (30% acetonitrile and 70% D_2O) δ: 8.05 (1H, s, H-2), 6.64 (1H, d, J=1.8 Hz, H-2′), 6.61 (1H, d, J=1.8 Hz, H-6′), 6.51 (1H, s, H-8), 3.75 (3H, s, OCH_3-5′), 3.74 (3H, s, OCH_3-4′), 3.69 (3H, s, OCH_3-6).

Irisflorentine (**5**): ESI-MS *m*/*z*: 387 $[M+H]^+$. UV λ_{max} (30% acetonitrile and 70% D_2O): 267, 323. ^{1}H-NMR (30% acetonitrile and 70% D_2O) δ: 8.02 (1H, s, H-2), 6.77 (1H, s, H-8), 6.73 (2H, s, H-2′, 6′), 6.03 (2H, s, –O–CH_2–O-6,7), 3.92 (3H, s, OCH_3-5), 3.76 (6H, s, OCH_3-3′,5′), 3.69 (3H, s, OCH_3-4′).

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