Characterization of Two Epimers, 4α and 4β, of a Novel Podophyllotoxin–4-*O*-(D)-6-Acetylglucopyranoside from *Podophyllum hexandrum* by LC–ESI-MS–MS

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Abstract

High-performance liquid chromatography (HPLC) with diode array detection interfaced to electrospray ionization (ESI) mass spectrometry (MS) is applied to identify the two epimers of a novel and minor constituent, podophyllotoxin–4-*O*-(D)-6-acetylglucopyraniside from high-altitude *Podophyllum hexandrum* for the first time. This is done by matching the structural information from the tandem MS data with the reported lignan markers. The results show that LC–MS–MS is the method of choice for fast detection and detailed chemical analysis of mixtures in the crude extracts of *Podophyllum*. The method can be employed in the absence of reference standards for the markers and is particularly useful in view of the scarcity of these rare chemical standards.

Introduction

Medicinal use of Podophyllum hexandrum Royale syn. P.emodi Wall (Himalyan Mayapple; family: Berberidaceae), a high-altitude plant species native to the alpine and sub-alpine areas of the Himalayas, dates back to ancient times (1). The plant has been described as "Aindri", a divine drug in the Indian traditional system of medicine (the Ayurveda) and has also been used in Traditional Chinese Medicine (2) for the treatment of a number of ailments. In the modern allopathic system of medicine, the plant products have been successfully used for the treatment of various disorders (3) such as monocytoid leukemia, Hodgkin's and non-Hodgkin's lymphomas, bacterial and viral infections (4,5), venereal warts (6), rheumatoid arthralgia associated with numbness of the limbs and pyogenic infection of skin tissue (2), AIDS-associated Kaposis sarcoma, and different cancers of the brain, lung, and bladder (7). The roots and rhizomes of P. hexandrum are known to synthesize a plethora of secondary metabolites besides podophyllotoxin and related aryltetrahydronephthalene lignans (1,2) with multifaceted pharmacological applications. Etoposide and teniposide, the two semi-synthetic glycoside derivatives of podophyllotoxin form an integral part of the therapeutic regimen used for chemotherapy (8) and have also triggered further studies in the design and synthesis of other potent anticancer compounds (9–11). As these lignans are highly toxic compounds, the use of Podophyllum hexandrum as an herbal medicine is potentially hazardous and needs to be carefully controlled. Analytical methods for characterization of the chemical constituents of the herb are thus necessary.

Methods for the identification of aryltetrahydronephthalene and related chemical marker lignans from P. hexandrum have rarely been reported. Difficulty in obtaining the reference standards for these markers is probably a major reason for identification of minor constituents. It is noted that aryltetrahydronephthalene lignans occuring in nature are all built around a common, basic skeleton and may show, under appropriate mass spectrometric (MS) condition, fragmentation pathways amenable to straightforward structural interpretation. It is thus worth exploring the possibility of identifying these minor constituents using their MS data with the aim of registering a chemo taxonomic profile, which could be diagnostic to herbs as well. The present paper reports the use of liquid chromatography (LC)–MS–MS for the identification of the two epimers, 4α and 4β, of a new compound, podophyllotoxin–4-O-(D)-6-acetylglucopyranoside (Compound **1** from Figure 1) from P. hexandrum along with many other known lignans from various fractions.

Experimental

Chemicals

Methanol and water were of high-performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany). Ammonium acetate and sodium acetate were of

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analytical grade (BDH, Glaxo, Haryana, India). Reagent-grade n-hexane, chloroform, and methanol (Ranbaxy, Gurgaon, India and Rankem, Mohali, India) were used for extraction.

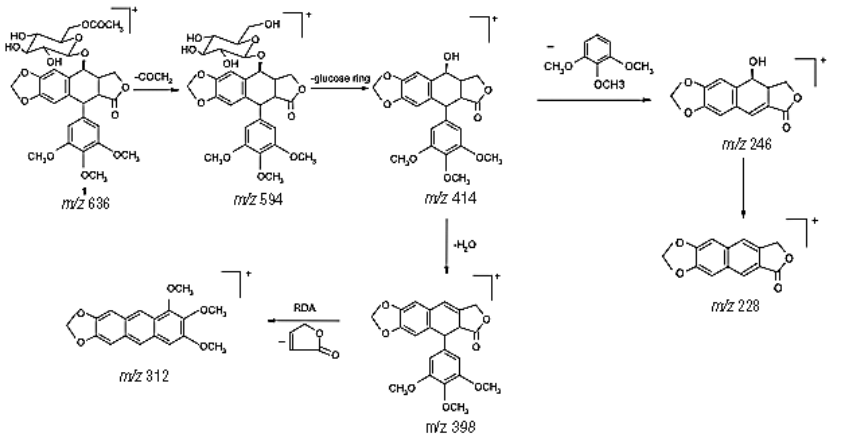

Figure 1. Fragmentation pathway for compound 1.

Plant materials

Six Podophylum hexandrum samples from different locations of Ladakh Valley, India were collected during April 2003 and identified by the Herbarium and Survey Deptartment of Botanical Sciences. A voucher sample was deposited in the Regional Research Laboratory repository in Jammu, India.

Apparatus

All experiments were performed using a Thermo-Finnigan MAT 800 pneumatically assisted electrospray triple-quadrupole MS (Cheshire, U.K.). A model pump no. 140C microgradient pump (Applied Biosystems, Foster City, CA) and a model 200 LC autosampler with a 2-µL injection loop were used (PerkinElmer, Ann Arbor, MI). The components of the plant extract were separated on a Luna RP-18 column (150- × 2-mm i.d., 3-µm particle size) with a safety guard (Phenomenex, Tor-

Table I. Lignans Identified Using LC–ESI-MS–MS Analysis in *Podophyllum hexandrum*

Fraction	Compound	t_R (min)	M+NH_4	Other major peaks
Standard 1	Podophyllotoxin	37.63	432.04	414.04, 396.93, 394.96, 352.97, 312.93
Standard 2	4'-Demethylpodophyllotoxin	28.91	418.04	400.04, 382.92, 298.92
Hexane fraction	4'-Demethylpodophyllotoxin glycoside	28.17	580.16	399.98, 382.98, 299.96, 298.90, 245.0
	Unidentified	28.97	636.17	594.11, 431.98, 412.00, 352.95, 312.9, 245.84
	4'-Demethylepipodophyllotoxin	31.83	418.01	382.96, 298.92, 245.89
	4'-Demethylpodophyllotoxin	31.88	418.01	382.96, 298.92, 245.89
	Podophyllotoxin glycoside	32.87	594.15	411.98, 352.97, 312.95
	Podophyllotoxin	36.46	432.04	396.93, 394.96, 352.97, 312.93
Chloroform fraction	4'-Demethylpodophyllotoxin	31.24	418.04	382.95, 298.92, 245.88
	Podophyllotoxin glycoside	32.39	594.18	432.05, 416.04, 397.01, 352.00, 312.86, 261.66
	epi-Podophyllotoxin	34.03	432.06	412.01, 52.94, 312.94, 397.00, 312.94
Alcoholic fraction	Unidentified	22.52	359.99	197.85, 179.84, 161.85, 448.91, 399.91
	4'-Demethylpodophyllotoxin glycoside	27.95	580.15	382.96, 339.08, 299.74, 298.93, 286.89
	4'-Demethylpodophyllotoxin	31.73	418.02	245.72, 231.02, 382.94, 298.89, 245.67
	Podophyllotoxin glycoside	32.86	594.09	541.98, 411.98, 396.94, 352.95, 312.95, 245.86.
	epi-podophyllotoxin	34.55	432.01	412.06, 352.96, 312.91, 118.83, 396.97
	Podophyllotoxin	36.40	433.05	394.94, 353.04, 312.95, 245.89
Hydro-alcoholic fraction	Unidentified	22.45	522.08	475.06, 458.09, 44.08, 359.98, 304.96, 265.91, 197.83, 179.82, 132.82, 302.87
	Unidentified	23.76	495.01	302.85, 286.86
	Unidentified	27.40	448.98	411.95, 352.96, 312.93, 286.92, 418.04
	Podophyllotoxin glycoside (iso)	29.14	594.18	382.95, 312.87, 245.75, 432.02, 411.97
	epi-podophyllotoxin	31.48	431.91	397.00, 352.94, 312.92
	Podophyllotoxin glycoside	32.69	594.13	245.87, 230.90, 213.86, 197.84, 136.89
	Unidentified	34.29	312.95	397.04, 263.89, 246.02
	Podophyllotoxin	36.18	432.02	

rance, CA) at 30°C. The mobile phase used was initially set as methanol–water (35:65). The composition was then changed to methanol–water (65:35) over a period of 60 min. The flow rate was 600 µL/ min and detection UV at λ = 290 nm.

LC–electrospray ionization-MS–MS conditions

The operating conditions were optimized for the LC–electrospray ionization (ESI)-MS–MS analysis of the aryltetrahydronaphthalene and related lignans as follows. The nebulizer, curtain, and collision gases were set at 1.25, 0.95, and 0.4 L/min, respectively. The gas was supplied from a liquid nitrogen tank with a head pressure of 50 psi. The ion spray voltage was 4800 V. The voltages at the orifice plate, focusing ring, deflector, and channel electron multiplier detector were 60, 350, –400, and 2100 V, respectively. Positive ions were scanned in the range 350–760 Da using a 10-ms dwell time and a 0.2-Da step size during scans.

Result and Discussion

The plant material was sequentially extracted with various solvents, such as n-hexane, chloroform, methanol, and aqueous methanol (40%) for HPLC, LC–MS, and LC–ESI-MS–MS profiling. In the LC separation it was found that a gradient of methanol and water containing 0.2% acetic acid was the optimal mobile phase. Water (A) and methanol (B) were varied as follows: 0–5 min (58% A and 42% B), 5–50 min (20% A and 80% B), 50–55 min (20% A and 80% B), 55–60 min (58% A and 42% B), and 60–65 min (58% A and 42% B) v/v at a flow rate of 1 mL/min.

Using the previously reported experimental conditions, the six P. hexandrum samples obtained from different sources from Ladakh valley (India) were analyzed. It was found that the patterns of their LC–MS reconstructed ion chromatograms were more or less similar except for some variation in the relative intensity of the peaks. By studying the fragmentation pattern as revealed in corresponding tandem mass spectra, a number of aryltetrahydronapthalene and related lignan marker compounds were identified in various extracts. The major fragment ions observed in the respective tandem mass spectra are summarized in Table I.

The compound identification was possible on the basis of fragmentation pathway (12,13). To ensure that the observed $[M+18]^+$ ion fragments of glycosylated compounds were indeed the ammoniated species, the herb extract was mixed with a diluent consisting of 10mM ammonium acetate in 0.1% acetic acid. The resulting MS–MS spectra showed an enhanced relative intensity of the $[M+18]^+$ peaks for the glycosylated compounds. Also, the subsequent MS–MS fragmentation patterns of the sodiated species and respective ammoniated species were the same (i.e., all involved the prior elimination of glucose and ammonium or sodium ions before going through fragmentation characteristics of the corresponding aglycone moiety) (14). Because of the high sensitivity of MS, this method was particularly

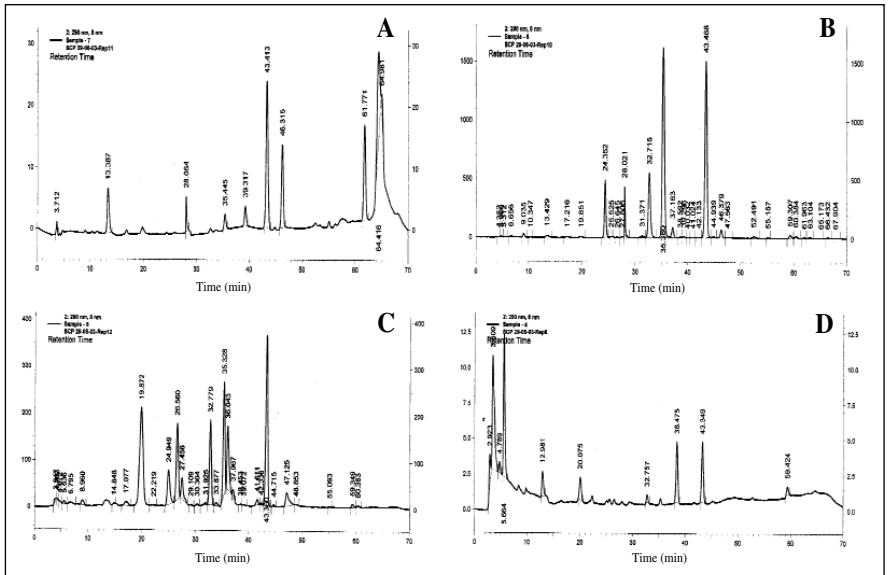

Figure 2. LC profiles of hexane (A), chloroform (B), methanol (C), and 40% aqueous–methanol extracts of high altitude *Podophyllum hexandrum* (D).

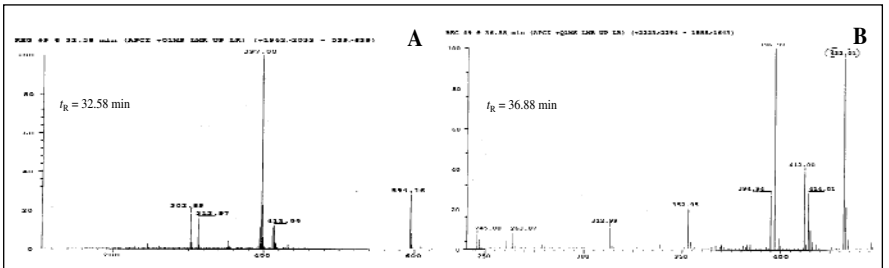

Figure 3. MS–MS of podophyllotoxin-β-D-glucopyranoside (A) and podophyllotoxin (B).

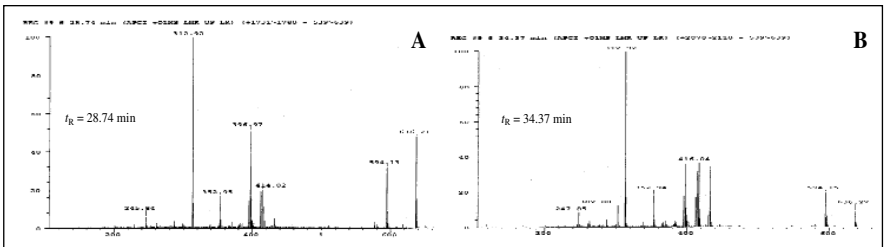

Figure 4. MS–MS of podophyllotoxin-4-*O*-β-D-6-acetylglucopyranoside (A) and podophyllotoxin-4-*O*-α-D-6-acetylglucopyranoside (B).

advantageous for the samples of limited quantity. Using this methodology, detailed structural information was obtained for lignans, lignan–glycosides, and acetyl glycosides in complex mixtures.

An integrated analytical system was used by incorporating HPLC separation (UV detection), MS, and MS–MS rapidly for providing profile and structural information useful for compound identification. Online HPLC separation afforded a profile of components in samples and their relative concentrations. HPLC provided a reproducible retention time using standardized HPLC conditions for the development of the lignan database. The reversed-phase HPLC conditions also provided a measure of the lipophilicity of each compound. UV spectra collected online provided evidence for general classification and substructures of each compound.

The ion spray (nebulizer assisted electrospray) interface generated reproducible abundant adduct ions $[M+NH_4]^+$, which were the base peak for all the lignans and their glycosides. The $[M+NH_4]^+$ ion provided reliable molecular weight confirmation from full scan MS as well as an abundant ion current, which is favorable for trace MS–MS analysis. The fragmentation patterns of podophyllotoxin and other well-characterized lignans were used as substructural templates for interpretation of unknown lignan structures by the association of specific product ions and neutral losses with specific substructures. This MS–MS comparative method is based on the premise that most of the compounds of interest contained in the extract sample would be expected to retain much of the original lignan structure, such as the aryltetrahydronapthalene substructure. Therefore, podophyllotoxin-related compounds would be expected to undergo fragmentation similar to that of podophyllotoxin. The MS–MS ion neutral losses observed in podophyllotoxin and in unknown lignans were evidence for common substructures, and differences were indicative of variance in these substructures. Structural data for some commonly known lignans from various fractions is provided in Table I.

A representative HPLC profile chromatogram with a UV detector (290 nm) of various fractions of Podophyllum rhizome is shown in (Figure 2). A large number of processed natural products from P. hexandrum were less complicated than typical crude biomass extracts.

Mass chromatograms (extracted ion current profiles) corresponding $[M+NH_4]^+$ ion of selected components indicate the specificity of LC–MS for molecular weight differentiation and determination of lignan component in a complex mixture. The difference between the molecular weight of podophyllotoxin, podophyllotoxin-4-O-β-D-glucopyranoside, and podophyllotoxin-4-O-β-(D)-6-acetylglycoside is indicative of substructural differences. A comparison of the full-scan ion spray mass spectrum of these lignan glycoside compounds demonstrates a molecular weight difference of 42 Da, which is commonly indicative of an acetyl substructural difference.

The structure of each lignan was proposed as based on a comparison of product ions and neutral losses as observed in the product ion spectrum, with product ion and neutral losses associated with a specific substructure of podophyllotoxin and podophyllotoxin-4-O-β-(D)-glucopyranoside (Figure 3).

The position of attachments of these substructures to the aryltetrahydronapthalene core was not determined using MS–MS but proposed on the basis of spectral comparison. The substructural analysis of a new compound, podophyllotoxin-4-O-β-(D)-6-acetylglucopyranoside, using LC–ESI-MS–MS is shown in (Figure 4) and a comparison with the substructural template (Figure 3A) confirmed compound **1** as shown from its fragmentation pattern (Figure 1).

The presence of a fragment ion at 594, along with molecular ions at m/z 636 with a difference of 42 Da, clearly indicates an addition of the acetyl group attached to the core podophyllotoxin glycosides. The presence of the product ions at m/z 594.13, 414.02, 396.97, 353.95, 313.92, and 247.85 is diagnostic of a podophyllotoxin and podophyllotoxin-4-O-β-glucopyranoside core. The observed molar mass of this compound (636 Da) differs from podophyllotoxin by 222 Da, fragment at 594 Da, with a 42 Da difference is because of deacetylation of the podophyllotoxin glycoside substructure.

The neutral loss of 222 Da from $[M+NH_4]$ ion to ion at m/z 414.2 further supports the assigned structure. The fragmentation of ammoniated glycoside fragment involved elimination of glucose and ammonia molecules to produce a fragment ion at m/z 397, which further breaks down through Retro Diels–Alder rearrangement after elimination of the lactone moiety to produce m/z 313 ion. Elimination of a trimethoxybenzene molecule (–168) from protonated molecules produces m/z 247, which on further elimination of water gave the m/z 228 ion. The position of the acetyl moiety at the primary hydroxyl group is based on the fact that a prominent peak such as M-42 (m/z 594) and very weak M-60 (m/z 558) were obtained, whereas in case of the acetyl group attached to the secondary hydroxyl with an available proton at the β-position should give a prominent peak at M-60 rather than M-42. Aryltetrahydornapthalene lignans undergo epimerization during glycosidation at the C-4 hydroxyl position. During LC–MS–MS profiling, two diastereoisomic glycosides at retention times (t_R) 28.74 and 34.37 min with same mass fragmentation pattern were identified.

Interestingly the 4-O-β-(D)-6-acetylglucopyranoside moves faster (t_R = 28.74 min) than the corresponding 4-O-α-(D)-6-acetylglucopyranoside (t_R = 34.37) because of the fact that the former has a 4β-glycoside attachment axial rather than equatorial, as is the case with 4α-epimer, as observed from their molecular models.

Conclusion

Two epimers, podophyllotoxin-4-O-α-(D)-6-acetylglucopyranoside and podophyllotoxin-4-O-β-(D)-6-acetylglucopyranoside, of a novel compound along with a number of known lignans have been characterized successfully using an LC–MS–MS method based on LC–ESI-MS–MS profiling. Our analytical strategy was applied to the identification of minor aryltetrahydronapthalene and related lignans, which are scarce and difficult to obtain.

Acknowledgments

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