DIETARY SUPPLEMENTS

Determination of the Appetite Suppressant P57 in *Hoodia gordonii* Plant Extracts and Dietary Supplements by Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC-MSD-TOF) and LC-UV Methods

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Hoodia gordonii **is traditionally used in South Africa for its appetite suppressant properties. P57AS3 (P57), an oxypregnane steroidal glycoside, is the only reported active constituent from this plant as an appetite suppressant. Effective quality control of these extracts or products requires rapid methods to determine P57 content. New methods of liquid chromatography/mass spectrometry (LC/MS) and LC-UV for analysis of P57 from** ***H. gordonii*** **have been developed. The quantitative determination of P57 was achieved with a Phenomenex Gemini (Torrance, CA) reversed-phase column using gradient mobile phase of water and acetonitrile, both containing 0.1% acetic acid. The method was validated for linearity, repeatability, and limits of detection and quantification. Good results were obtained in terms of repeatability (relative standard deviation <5.0%) and recovery (98.5–103.5%). The developed methods were applied to the determination of P57 for** ***H. gordonii*** **plant samples, one related genus (*Opuntia ficus-indica*), and dietary supplements that claim to contain** ***H. gordonii*****.**

Hoodia gordonii, Family Asclepiadaceae, is a succulent plant found in summer rainfall regions of the Kalahari Desert in South Africa, Namibia, and Botswana (1–2). There are over 20 reported species of the genus *Hoodia*; however, because of its anorectic activity (2, 3) *H. gordonii* is the only sought-after species for trade. Various uses of *Hoodia* have been reported in African folklore for centuries, such as treatment of abdominal cramps, hemorrhoids, tuberculosis, and diabetes, and as an aphrodisiac (3, 4). The Xhomani San Bushmen in the Kalahari Desert would eat fresh *H. gordonii* to suppress their appetite and aid in hydration while on long hunting trips (5). P57, an oxypregnane steroidal glycoside, is the only reported active constituent from this plant and acts as an appetite suppressant (6–9). It has been shown to increase the adenosine triphosphate ATP content in hypothalamus neurons that regulate the food intake (5). The structure and UV spectrum of P57 are shown in Figures 1 and 2. Phytopharm, Inc. (Godmanchester, UK) patented the active appetite suppressant extracts of *H. currorii*, *H. gordonii*, and *H. lugerdii* (5). The patent was licensed to Phytopharm, and the active constituent from *H. gordonii* was commonly named P57. P57 is also known as P57AS3 (10, 11). Apart from the anorectic activity, it is also used to prevent aspirin-induced gastric damage (11) and has antidiabetic activity (4).

Because *H. gordonii* is native to the Kalahari Desert, its growth outside its natural environment is extremely difficult. As a succulent, it thrives in extremely hot weather and takes decades to mature. Due to the plant's limited area of growth and its slow cycle of maturation, *H. gordonii* is limited in its supply. *H. gordonii* is now listed as an endangered species and its export out of South Africa is strictly controlled by the South African government (2). At the same time, the demand for weight loss products containing *H. gordonii* is high and increasing in the United States. Because the supply of authentic *H. gordonii* cannot match the demand for product, the adulteration of the product then becomes a possibility. One possible adulterant may be prickly pear cactus (*Opuntia ficus-indica*), which grows quickly and in abundance in North and Central America. However, there has been no appetite-suppressing activity associated with this species.

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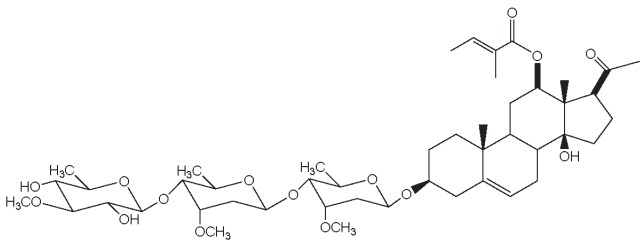

Figure 1. Structure of P57.

Because adulteration of products claiming to contain *H. gordonii* is possible, developing techniques for product analysis becomes crucial for product validity and safety. Currently, there are no analytical methods reported in the literature for the determination of *H. gordonii*. As an effort to develop methods for quality control and authentication of *H. gordonii*, the active appetite suppressant (P57) was isolated. Little chemistry of the plant is known, and an in-depth study is in progress for isolating other chemical constituents and developing a chemical fingerprinting technique. To determine the content of P57, a marker compound found in *H. gordonii*, analytical methods have been developed. The present study was undertaken to confirm the absence or presence of P57 in various plant samples and commercial products claiming to contain *H. gordonii*. In the present work, a liquid chromatography (LC)-UV method and a more sensitive LC/mass spectrometry (MS) method were developed. Authentic *H. gordonii* was obtained from Missouri Botanical Garden, St. Louis, MO. This study also analyzed dietary supplements claiming to contain *H. gordonii*, and compared *O. ficus-indica* to *H. gordonii*.

METHOD

Instrumentation and Chromatographic Conditions

LC/MS time of flight (TOF).—The liquid chromatograph used was an Agilent Series 1100 comprising the following modular components: quaternary pump, a vacuum solvent microdegasser, an autosampler with 100-well tray, and an online diode array detector (DAD). The analysis was performed on an Agilent Series 1100 SL equipped with an electrospray ionization (ESI) source. All acquisitions were performed under positive ionization mode with a capillary voltage of +4000 V. Nitrogen was used as nebulizer gas (30 psig) as well as drying gas at 10 L/min at drying gas temperature of 325°C. Data acquisition and processing were performed with the software Analyst^{TM} QS (Agilent Technologies, Palo Alto, CA).

Separation was achieved on a Gemini C18 column (Phenomenex, Torrance, CA), 150 × 4.6 mm id; 5 μm particle size. The column was equipped with a 2 cm LC-18 guard column (Supelco, Bellefonte, PA). The mobile phase consisted of (A) water–0.1% acetic acid; (B) acetonitrile 0.1% acetic acid at a flow rate of 0.5 mL/min; a gradient elution was as follows: 50% A:50% B to 100% B in 25 min. Each run was followed by a 5 min wash with 100% acetonitrile and an equilibration period of 15 min.

LC-UV analysis.—The high-performance liquid chromatography (HPLC) system consisted of Model 6000A pumps, Model U6K injector, Model 680 automated gradient controller, Model 996 photodiode array detector (all from Waters Corp., Milford, MA), and a computerized data station equipped with Waters Millennium software. Separation was achieved on a Gemini C18 column (150 × 4.6 mm id; 5 μm particle size; Phenomenex) and operated at 30°C. The column was equipped with a 2 cm LC-18 guard column (Supelco). The mobile phase consisted of (A) water and (B) acetonitrile, both containing 0.1% acetic acid that was applied in the following gradient elution: 0 min, 80% A:20% B in next 35 min to 100% B. Each run was followed by a 5 min wash with 100% acetonitrile and an equilibration period of 15 min. The flow rate was adjusted to 1.0 mL/min. P57 was detected at 220 nm.

Chemicals

The standard compound (P57) was isolated at the National Center for Natural Products Research (NCNPR), University of Mississippi, University, MS; the identity and purity were confirmed by thin-layer chromatography (TLC) and HPLC methods, by the analysis of the spectral data [infrared (IR) 1D and 2D nuclear magnetic resonance (NMR) high-resolution (HR) ESI-MS] and comparison with published spectral data (12).

Acetonitrile and glacial acetic acid were of HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ). Water for the HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA).

Dietary supplements claiming to contain *H. gordonii* were purchased online. Different *H. gordonii* plant samples were obtained from Missouri Botanical Garden. *Opuntia ficus-indica* was obtained from local stores. Voucher and retain specimens of all samples are deposited at NCNPR.

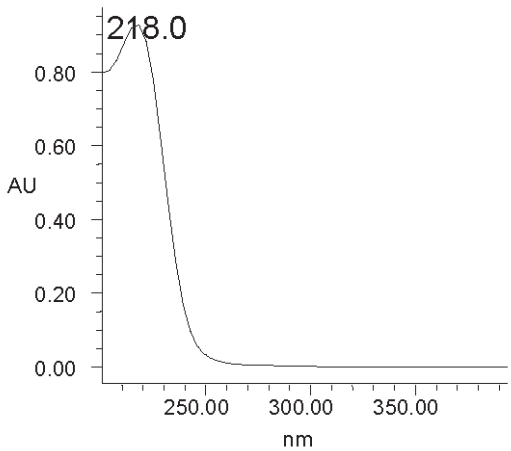

Figure 2. UV spectrum of compound P57.

Table 1. Calibration data limits of detection (LOD) and quantification (LOQ) by LC/MS and LC-UV methods

Parameter	LC/MS	LC-UV
Sample No.	1	1
Compound	P57	P57
Regression equation	y = 6.83e + 004X	y = 4.53e + 003X
Correlation coefficient, r^2	0.99997	0.99992
LOD, ng/mL	10	100.0
LOQ, ng/mL	25	500.0
Exact mol. wt.	878.5806	

Standard Solutions for Accuracy and Precision Determination

An individual stock solution of standard compound (P57) was prepared at a concentration of 1.0 µg/mL in methanol. The calibration curves were prepared at 7 different concentration levels, in triplicate once each day, and were used to assess accuracy and precision of each assay method. The range of the calibration curves was approximately 25–1000 ng/mL. Table 1 shows the LC/MS and LC-UV calibration data and the calculated LOD for each method.

Sample Preparation

Dry *H. gordonii* samples (0.5 g) or an adequate amount of powdered tablet or capsule were sonicated in 2.5 mL methanol for 20 min followed by centrifugation for 15 min at 9000 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated 3 times, and respective supernatants were combined. The final volume was diluted to volume with methanol and mixed thoroughly.

Prior to injection, an adequate volume (ca 2 mL) was passed through a 0.45 µm nylon membrane filter. The first 1.0 mL was discarded, and the remaining volume was collected in an HPLC sample vial. Each sample solution was injected in triplicate.

Accuracy

A recovery experiment was performed to confirm the accuracy of the method. Samples (HP-2, HG-2, HG-4, and HG-5) were spiked with 1.0 mL standard stock solution and then extracted and analyzed under optimized conditions. The recovery rates were in the range of 98.5–103.5% for compound P57.

Ruggedness

Intra- and interday assay.—Precision of the method was determined by analyzing 5 individual samples of specimens (HP-2, HG-2, HG-4, and HG-5) on 3 consecutive days. The samples were extracted and assayed under optimized conditions (Table 2).

Results and Discussion

Chromatographic Conditions

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C18 column. The different columns tried were Synergi Max-RP 80 A, Aqua C18, Gemini C18, and Luna C18. The best results were observed with the Gemini C18 column using water and acetonitrile, both containing 0.1% acetic acid as mobile phase. Variation of the column temperature between 25–40°C did not cause significant change in the resolution; however, changes in retention time were observed. The column was used at 30°C at a flow rate of 0.5 mL/min (LC/MS) and 1.0 mL/min for the LC-UV method. Thus in order to obtain good detection sensitivity and resolution of peak, the flow rate was reduced to 0.5 mL/min in the LC/MS method and the solvent gradient was modified.

Accuracy, Precision, and Linearity

The 7-point calibration curves for P57 showed a linear correlation between concentration and peak area. The method was validated in accordance with the U.S. Pharmacopeia (USP) by determining several analytical and statistical parameters. Calibration data (Table 1) indicated the linearity (r^2 > 0.999) of the detector response for all standard compounds from 25 to 1000 ng/mL. The LODs were found to be 10.0 ng/mL (LC/MS method) and 100 ng/mL (LC-UV

Table 2. Intra- and interday precision of samples (HP-1, HG-2, HG-4, and HG-5) assayed under optimized conditions for compound P57 by LC-UV method

	Intraday (n = 5)			
Compound[a]	Day 1	Day 2	Day 3	Interday (n = 3)
HP-2*	0.0468 (3.82)[b]	0.0494 (4.66)	0.0448 (3.99)	0.047 (3.13)
HG-2**	—	—	—	—
HG-4**	0.1688 (1.79)	0.1702 (1.78)	0.175 (1.94)	0.169 (1.53)
HG-5**	0.0047 (2.73)	0.0050 (3.86)	0.0049 (4.30)	0.0048 (2.17)

[a] Values in mg/100 mg of plant sample (*) or capsule content weight (**).

[b] Relative standard deviation given in parentheses.

Table 3. Intra- and interday precision of samples (HP-2, HG-2, HG-4, and HG-5) assayed under optimized conditions for compound P57 by LC/MS method

	Intraday (n = 5)			
Compound[a]	Day 1[b]	Day 2	Day 3	Interday (n = 5)
HP-2*	0.048 (2.08)	0.046 (2.82)	0.047 (3.36)	0.048 (2.08)
HG-2**	—	—	—	—
HG-4**	0.171 (0.93)	0.169 (1.29)	0.175 (1.25)	0.171 (0.93)
HG-5**	0.0049 (2.49)	0.0046 (3.61)	0.0047 (3.79)	0.0049 (2.49)

[a] Values in mg/100 mg of plant sample (*) or capsule content weight (**).

[b] Relative standard deviation given in parentheses.

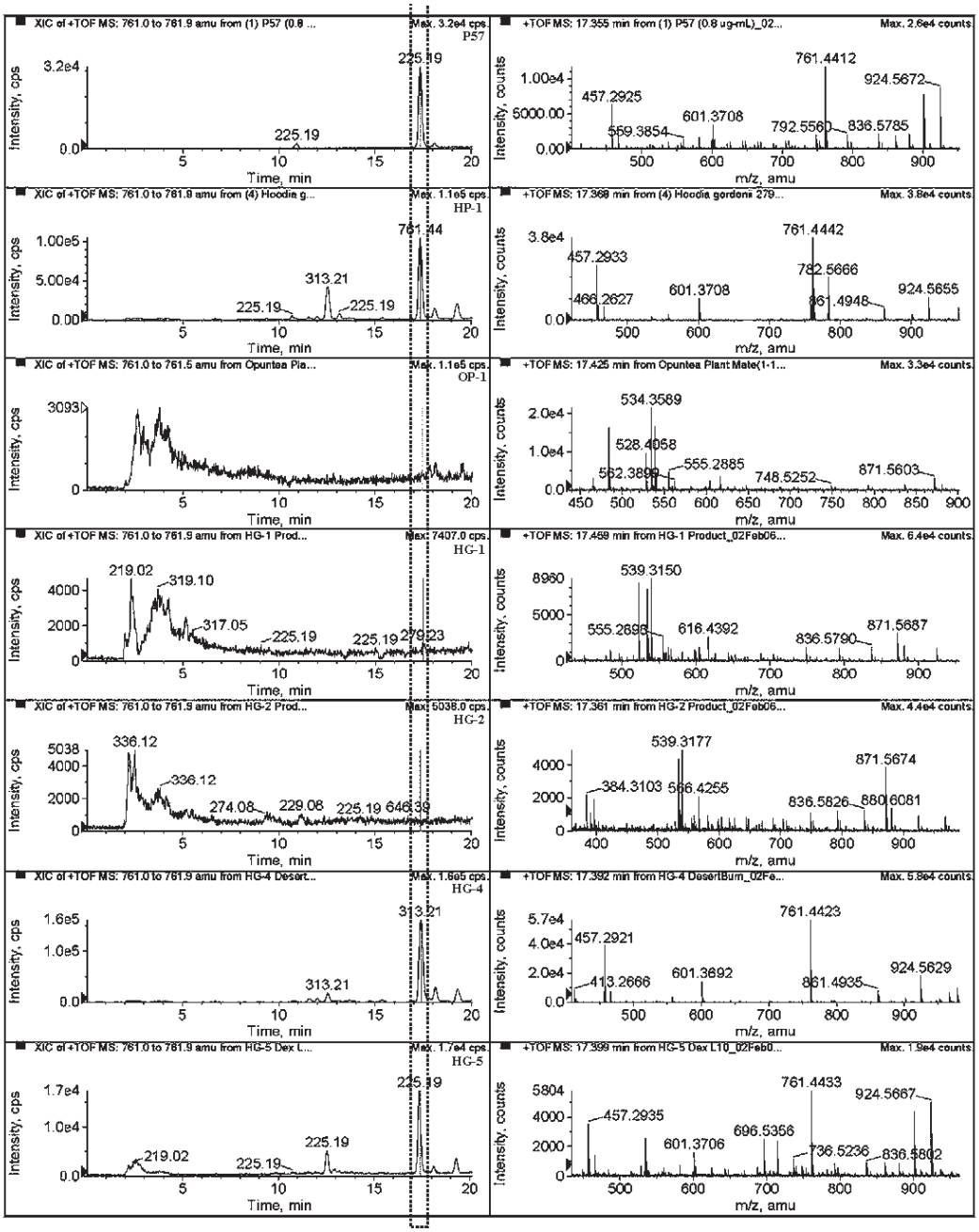

Figure 3. XIC and mass spectra of compound (P57) extracted from plant samples (HP-1, OP-1) and dietary supplements (HG-1, HG-2, HG-4, and HG-5) claiming to contain *H. gordonii*.

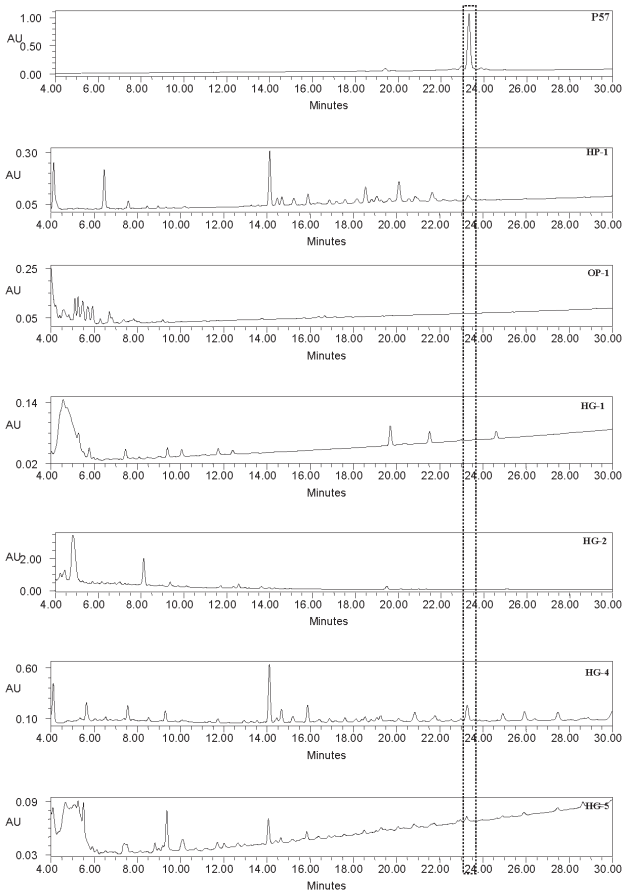

Figure 4. LC-UV chromatograms of P57, *H. gordonii* plant samples, and dietary supplements that claim to contain *H. gordonii*.

method). Accuracy of the method was confirmed by performing a recovery experiment. Samples (HP-2, HG-2, HG-4, and HG-5) were spiked with known amounts of the standard compound at 2 different concentrations (0.5 and 1.0 μg/mL), extracted, and analyzed. Compared to the theoretical amounts, recoveries of 98.5–103.5% for compound P57 were obtained. These data not only confirm the accuracy of the method but also the integrity of the extraction procedure. The proposed extraction procedure was evaluated for its efficiency prior to the analysis of plant materials and commercial products. One sample (HP-2) was extracted 5 times with 3.0 mL methanol and the supernatant obtained after each extraction step was separated and analyzed by HPLC. A minimum of 98.7% of P57 compound was in solution after repeating the procedure 4 times. Thus, a 4-fold extraction was considered to be exhaustive. All standards and samples were injected in triplicate. Multiple injections showed that the results are highly reproducible and showed low standard error. Intra- and interday (Tables 2 and 3) variation of the assay was determined and shown to be <5.0%, with a maximum relative standard deviation (RSD) of 4.66. It was performed 5 times on 3 different days, and each concentration point was injected in triplicate. Peak symmetry was good while retention time was retained at approximately 23.29 min. Peak purity and identity were verified by studying photodiode array (PDA) and MS data, as well as by spiking samples with reference compound. No indications of impurities were found. The analytical parameters such as accuracy, precision, linearity, and LOD are important for the assessment of the quality level of dietary supplements.

Analysis of Plant Samples and Dietary Supplements

The LC/MS data (Figure 3) and the LC-UV chromatograms (Figure 4) show the presence or absence of P57 in *H. gordonii* plant sample (HP-1), *O. ficus-indica* plant sample (OP-1), and dietary supplements. The methods were applied for quantification of various plant samples and commercial products. The plant extracts (HP-1, HP-2, and OP-1) and commercial products (HG-1–HG-10) were analyzed using LC-UV and LC/MS, and the concentration of P57 is shown in Table 4. The content of P57 detected in *H. gordonii* plant samples was 0.0052% (LC/MS) and 0.0051% (LC-UV) for HP-1, and 0.048% (LC/MS) and

Table 4. P57 content by LC/MS and LC-UV in various *H. gordonii* plant extracts, related genus (*O. ficus-indica*), and dietary supplements that claim to contain *H. gordonii*

	P57 content, %	
Species	LC-UV	LC/MS
Plant samples		
H. gordonii (HP-1)	0.0051	0.0052
H. gordonii (HP-2)	0.047	0.048
O. ficus-indica (OP-1)	ND[a]	ND
Capsules		
H. gordonii (HG-1)	ND	ND
H. gordonii (HG-2)	ND	ND
H. gordonii (HG-3)	ND	ND
H. gordonii (HG-4)	0.169	0.171
H. gordonii (HG-5)	0.0048	0.0049
Tablets (Batch-1)		
H. gordonii (HG-6)	ND	ND
Tablets (Batch-2)		
H. gordonii (HG-7)	ND	ND
Tablets (Batch-3)		
H. gordonii (HG-8)	ND	ND
Tablets (Batch-4)		
H. gordonii (HG-9)	ND	ND
Tablets (Batch-5)		
H. gordonii (HG-10)	ND	ND

[a] ND = Not detected.

0.047% (LC-UV) for HP-2. P57 was not detected in *O. ficus-indica* plant extract (OP-1). Ten commercially available dietary supplements were tested (HG-1–HG-10). Two products (HG-4 and HG-5) contained 0.17 and 0.0048% P57, respectively, by the LC-UV method, and 0.17 and 0.0049% by the LC/MS method. Four products (HG-1–HG-3 and HG-6) did not show the presence of P57 by the 2 methods (LC/MS and LC-UV). HG-6–HG-10 were 5 different batches of 1 product (HG-6). The LC/MS method (LOD = 10 ng/mL) was shown to be more sensitive than the LC-UV method (100 ng/mL).

The LC/MS analysis (Figure 3) revealed that P57 had a molecular peak at 924.56 $[M+2Na]^+$ and a base peak at 761.44 $[M-117]^+$, accounting for the loss of the tigloyl moiety and ·OH. No interfering peaks were found at the retention time of interest. By the LC-UV method, the identification of the P57 in *H. gordonii* samples and dietary supplements was based on the retention times and the comparison of UV spectra or by spiking the extracts with reference compound, P57. The UV spectrum and mass for P57 (Figure 2) and its presence in other samples identified were the same.

Conclusions

H. gordonii has been traditionally used in South Africa for its appetite-suppressant activities. New LC-UV and more sensitive LC/MS methods have been developed. The developed methods permitted the quantitative determination of P57 in *H. gordonii* plant samples as well as in dietary supplements that claim to contain *H. gordonii*.

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