Simultaneous Determination of Four Major Steroidal Saponins in Seven Species of *Dioscore*a L. by HPLC-ELSD

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Abstract: **Objective** To control the quality of the species in *Dioscorea* L. better. **Methods** An HPLC-ELSD method was developed for the first time to simultaneously determine four bioactive ingredients: dioscin, gracillin, protoneodioscin, and protoneogracillin in 31 samples belonging to seven species of *Dioscorea* L. from different areas. The column was an Inertsil HILIC (250 mm × 4.6 mm, 5 μm). The separation was carried out with a gradient program. The mobile phase was acetonitrile-water at a flow rate of 0.8 mL/min. **Results** The standard curve was rectilinear in the range of 0.464－12.97 μg (r = 0.9969) for dioscin, 0.310－7.09 μg (r = 0.9953) for gracillin, 0.469－11.66 μg (r = 0.9970) for protoneodioscin, and 0.276－6.87 μg (r = 0.9992) for protoneogracillin. The recoveries of the markers were 98.1%, 100.1%, 97.2%, and 96.4%, respectively. The contents of the four components were quite different among the seven species of *Dioscorea* L. **Conclusion** The proposed HPLC-ELSD method is convenient, fast, accurate, and applicable for simultaneous analysis of multiple bioactive components of species in *Dioscorea* L. for quality control, which could facilitate discovering new natural resources of steroidal saponin.

Key words: dioscin; *Dioscorea* L.; gracillin; HPLC-ELSD; protoneodioscin; protoneogracillin

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Introduction

Dioscorea spongiosas J. Q. Xi, M. Mizuno et W. L. Zhao (*Mianbixie*), *D. hypoglauca* Palibin (*Fenbixie*), *D. nipponica* Makino (*Chuanlongshuyu*), *D. tokoro* Makino (*Shanbixie*), *D. tenuipes* Franch. et Sav. (*Xibingshuyu*), *D. gracillima* Miq. (*Xianxishuyu*), and *D. zingiberensis* C. H. Wright (*Dunyeshuyu*) are members of *Dioscorea* L., which are abundant in nature and distributed widely in Zhejiang, Anhui, Jiangxi, and Fujian Provinces of China. Some of them such as *D. spongiosas*, *D. hypoglauca*, and *D. nipponica*, are commonly used as traditional Chinese medicines (TCMs) with their rhizomes (Pharmacopoeia Committee of P. R. China, 2010). In previous phytochemical studies, more than 50 compounds of steroidal saponins from plants of *Dioscorea* L. have been isolated (Tang, Yang, and Pan, 2007). Extensive phytochemical and pharmacological studies have shown that steroidal saponins have biological activities contributing to the efficacy of the herb (Liu *et al*, 2010). Unfortunately, few studies on simultaneous determination of the multi-compounds in plants of *Dioscorea* L. were reported, though quantitative analysis of these constituents is of great significance for searching new natural resources of steroidal saponin (Tang, Yang, and Pan, 2007; Wang, Liu, and Yin, 2007). The methods for determination of steroidal saponins in plants of *Dioscorea* L. have been developed using HPLC and TLC scanning methods (Qin, Jia, and Sun, 2007; Liu, Wang, Lu, 2008; Liu, Wang, and Wen, 2006; Yin, Kouda, and Tezuka, 2003; 2004; Mo and Yang, 2003; Mo, 2003). Moreover, one or two constituents could not be responsible for overall pharmacological activities in plants of *Dioscorea* L. The four marked components, dioscin, gracillin, protoneodioscin, and protoneogracillin, in plants of *Dioscorea* L. had too weak responses and too many interfering peaks to be detected simultaneously with UV detector. Thus it is impossible to simultaneously determine these bioactive components using UV detection. In the present study, a sensitive and simple HPLC-ELSD method was established for the quality control

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of seven species of *Dioscorea* L. and successfully applied for the assessment of 31 commercial samples.

Materials and methods

Chemicals and reagents

HPLC grade acetonitrile was purchased from Tedia (Fairfield, OH, USA). Ultrapure water was prepared from a Milli-Q purification system (Millipore Co., France) and ethanol (AR) was obtained from Hangzhou Shuanglin Chemical Company (Hangzhou, China). Standard substances of dioscin, gracillin, protoneodioscin, and protoneogracillin with more than 98% of purity were all purchased from Academy of Military Medical Sciences (Beijing, China).

Materials

Crude drug samples were obtained from corresponding Institutes for Food and Drug Control in China or were collected by the author. All samples were identified by Zhejiang Institute of Drug Control. The voucher samples were deposited.

Sample preparation

The raw herbs were pulverized into powders and dried to constant weight before use. The herb powders (300 mg) were placed in a conical flask with stopper, accurately 25 mL of 70% ethanol was added and weighed in the stoppered closely conical flask. Ultrasonicate (300 W, 45 kHz) for 30 min, stand to cool, and weigh again. Replenish the loss of the weight with ethanol and mix well. The solution obtained was filtered through a 0.45 μm filter and 10 μL of the filtrate was injected into the HPLC instrument for analysis.

HPLC apparatus and conditions

Analyses were performed on an Agilent 1100 Series HPLC instrument equipped with a quaternary pump, an autosampler, a column compartment, and Agilent Chemstation of LC and LC-MS systems (California, USA), coupled with an Alltech ELSD 2000 detector. Samples were separated on an Inertsil HILIC column (250 mm × 4.6 mm, 5 μm, Shimadzu) by using acetonitrile-water as the mobile phase with a gradient elution, in which acetonitrile was decreased from 90% to 78% within 30 min. The total acquisition time was 30 min. The mobile phase flow rate was 0.8 mL/min and 10 μL of sample solution was injected in each run. The column temperature was set at 30 ℃.

Detection conditions

For ELSD detection, the carrier gas was nitrogen (99.999%), the drift tube temperature was set at 90 ℃, and the gas flow rate was 2.5 L/min.

Results

Extraction

Conditions for extraction of dioscin, gracillin, protoneodioscin, and protoneogracillin from plants of *Dioscorea* L. were optimized by single factor experiments. Ethanol, 70% ethanol, and 40% ethanol used as extraction solvent were evaluated by concentrations of the four marked compounds. The best solvent was 70% ethanol, which enabled higher concentrations of the four marked compounds than those by ethanol and 40% ethanol. Methanol should be avoided, which can make protoneodioscin and protoneogracillin methylation. Reflux method can make protoneodioscin and protoneogracillin unstable, and ultrasonic method was appropriate for the extraction of the active constituents in plants of *Dioscorea* L. due to its higher yield of the four marked compounds than those by reflux method. Extraction time and solvent volume for extraction, which may affect medicine quality, were also optimized. The results suggested that all marked compounds were almost the same yield with extraction time of 30 and 45 min but higher than that of 15 min, and the same yield with extraction solvent volume of 25 and 35 mL but higher than that of 15 mL. From experiments it could be concluded that the best extraction process was ultrasonic method by using 25 mL of 70% ethanol as extraction solvent for 30 min.

Optimization of separation conditions

In this study, the four marked components from the plants of *Dioscorea* L. could not be separated effectively by using octadecylsilane bonded silica gel column and the isocratic mobile solvents. In order to find an easy way to analyze the components, we employed a hydrophilic interaction column chromatography and a gradient solvent system (acetonitrile and water), which can effectively separate the four makers simultaneously. Optimized chromatographic conditions were achieved after several trials with acetonitrile, acetic acid, and water in different proportions as mobile phase. The solution obtained from each sample of *Dioscorea* L. was injected into the instrument and the peaks in the chromatograms obtained were identified by

t_R in comparison with those of reference standards, and also by the method of standard addition to the sample. The representative chromatograms of the samples were shown in Fig. 1. From that, it could be concluded that all reference compounds were eluted with highly symmetrical peaks under the condition.

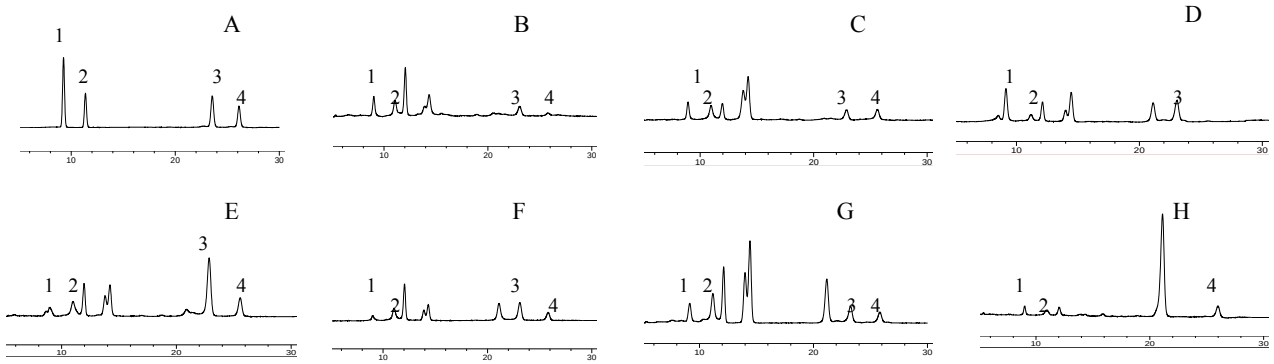

Fig. 1 Representative HPLC chromatograms of the standards

A: Peaks 1, 2, 3, and 4 were dioscin, gracillin, protoneodioscin, and protoneogracillin, respectively; B: *D. spongiosas*; C: *D. hypoglauca*; D: *D. nipponica*; E: *D. tokoro*; F: *D. tenueipes*; G: *D. gracillima*; H: *D. zingiberensis*

In this study, both HPLC-ELSD and HPLC-UV analyses of the plants in *Dioscorea* L. were performed. The four marked components from the plants of *Dioscorea* L. had too weak responses and too many interfering peaks to be detected simultaneously with UV detection. Thus it is impossible to simultaneously determine these bioactive components using UV detection. All the four components detected simultaneously by ELSD detector were indicated in Fig. 1. Therefore, ELSD detection should be accepted as a tool in the quality control for the plants of *Dioscorea* L.

System suitability test

System suitability was tested by performing five replicate injections of the standard solutions and determining peak areas, theoretical plate number (*N*), and asymmetry factor (*A*s) for the analytes of interest. The relative standard deviations (RSD) of these properties were used as indicators of system suitability. For pharmaceutical applications the RSD of peak area should be less than 2.0% for all the analytes of interest. The mean values of *N* for the four compounds were 11 985, 16 637, 38 657, and 45 451, respectively, and the *A*s values were 0.95, 0.97, 0.99, and 1.01, respectively. The RSD of the peak areas ranged from 1.10% to 1.69%. These results showed that the proposed method could meet the requirements.

Validation of the method

The assay linearity was determined by the analysis of five different concentrations of the standard solutions. The linear regression data were estimated and reported in Table 1. All the calibration curves were linear within the test range and followed the equation of the type of $y = ax + b$ with high correlation. The linearity of the plots was over 0.995.

Table 1 Calibration curves for the four compounds

Analytes	Calibration	*r*	Linear range / μg
dioscin	$y = 1.032x + 1.875$	0.9969	0.464—12.970
gracillin	$y = 1.072x + 1.848$	0.9953	0.310— 7.086
protoneodioscin	$y = 1.393x + 1.448$	0.9970	0.469—11.660
protoneogracillin	$y = 1.457x + 1.619$	0.9992	0.276— 6.866

y and *x* denote the logarithmic value of peak area and concentration, respectively

In order to test the repeatability, six sample solutions of *Dioscorea* L. (*D. gracillima*, Zhejiang Chun'an) were prepared. The contents of dioscin, gracillin, protoneodioscin, and protoneogracillin were calculated and the RSD values were 2.1%, 2.7%, 2.3% and 2.7%, respectively. The limit of detection (LOD) was determined as the concentration resulting in a peak height greater than three times of the baseline noise level (S/N > 3). In this study, LOD values of dioscin, gracillin, protoneodioscin, and protoneogracillin were also determined with the results of 17, 25, 110, and 96 ng, respectively. Recovery of the four compounds was determined by adding the standards to crude drug powder which was treated according to the procedure described above. Results indicate that the accuracy and precision of the proposed method are sufficient for determination of the four compounds in the crude drugs (Table 2).

Table 2 Recoveries of the four compounds (n = 6)

Compounds	Contained / mg	Added /mg	Found / mg	Recovery / %	RSD / %
dioscin	1.536	1.645	3.151	98.1	2.8
gracillin	2.798	2.901	5.703	100.1	2.6
protoneodioscin	3.011	2.751	5.686	97.2	2.0
protoneogracillin	1.779	1.611	3.331	96.4	2.3

A sample solution stored in brown bottle was injected to apparatus respectively when placed for 0, 1, 8, 12, and 24 h. The RSD value of peak area for each compound was calculated. The RSD values were less than 2.0%.

Application

A diluted extraction solution obtained from each sample of *Dioscorea* L. was injected into the instrument and the peaks in the chromatograms obtained were identified by comparison of t_R with those of the standards. The amounts of the four compounds in the samples were calculated. The results were shown in Table 3.

Discussion

Data obtained from 31 samples suggested that all the four compounds were found in *D. spongiosas*, *D. hypoglauca*, *D. tokoro*, *D. tenueipes*, and *D. gracillima.* Dioscin, gracillin, and protoneodioscin were found in *D. nipponica.* Dioscin, gracillin, and protoneogracillin were found in *D. zingiberensis.* The majority of the components

Table 3 Contents of the four compounds in samples of *Dioscorea* L.

Species	Collection place	Dioscin / %	Gracillin / %	Protoneodioscin / %	Protoneogracillin / %	Total / %
D. spongiosas	Zhejiang	0.26	0.25	0.37	0.11	0.99
D. spongiosas	Quzhou	0.44	0.23	0.58	0.23	1.48
D. spongiosas	Lin'an, Zhejiang	0.36	0.51	1.19	0.23	2.29
D. spongiosas	Zhejiang	0.47	0.46	0.71	0.19	1.83
D. spongiosas	Hangzhou	0.42	0.20	0.41	0.31	1.34
D. spongiosas	Lin'an, Zhejiang	0.40	0.21	0.75	0.16	1.52
D. gracillima	Chun'an, Zhejiang	1.06	1.89	2.26	1.32	6.53
D. gracillima	Cuichang, Zhejiang	0.41	1.46	1.15	0.63	3.65
D. gracillima	Shaoxing, Zhejiang	0.52	0.75	1.31	0.84	3.42
D. gracillima	Shengzhou, Zhejiang	0.69	0.62	2.51	1.10	4.92
D. hypoglauca	Bozhou, Anhui	1.01	0.54	4.38	0.27	6.20
D. hypoglauca	Xianju, Zhejiang	0.73	0.57	1.16	0.88	3.34
D. hypoglauca	Xianju, Zhejiang	1.14	0.59	2.02	0.45	4.20
D. hypoglauca	Xianju, Zhejiang	0.62	2.80	1.44	0.88	5.74
D. hypoglauca	Xianju, Zhejiang	1.32	0.70	3.40	0.69	6.11
D. hypoglauca	Bozhou, Anhui	1.31	0.60	4.80	0.64	7.35
D. hypoglauca	Bozhou, Anhui	0.31	0.91	2.00	0.57	3.79
D. hypoglauca	Jingning, Zhejiang	0.77	0.80	0.92	0.70	3.19
D. hypoglauca	Bozhou, Anhui	0.47	1.81	1.27	0.81	4.36
D. hypoglauca	Beijing	1.88	0.65	2.26	0.24	5.03
D. nipponica	Shenzhen	1.15	1.25	1.64	—	4.04
D. nipponica	Shenzhen	1.34	1.10	1.36	—	3.80
D. nipponica	Shenzhen	2.18	2.53	0.77	—	5.48
D. nipponica	Shenzhen	1.28	0.96	2.00	—	4.24
D. nipponica	Shenzhen	0.93	0.34	1.26	—	2.53
D. nipponica	Shenzhen	1.55	1.16	1.12	—	3.83
D. nipponica	Beijing	1.39	0.72	3.42	—	5.53
D. zingiberensis	Xianju, Zhejiang	0.21	0.66	—	0.61	1.48
D. zingiberensis	Bejing	0.33	0.38	—	0.90	1.61
D. tenuipes	Zhejing	0.24	0.96	1.78	0.72	3.70
D. Tokoro	Jingning, Zhejing	0.59	0.79	1.28	0.82	3.48

in *D. hypoglauca*, *D. tokoro*, *D. tenueipes*, *D. nipponica*, and *D. gracillima* have higher concentrations than those in *D. spongiosas* and *D. zingiberensis.* The samples of *D. nipponica* were provided by Shenzhen Institute for Food and Drug Control. Since the samples produced from different places, the amounts of the compounds in *D. nipponica* could be different.

Conclusion

In conclusion, a simple, sensitive, precise, and reproducible HPLC method could make it possible to simultaneously determine four compounds in plants of *Dioscorea* L. The good linearity of the calibration graphs could enable quantitative assay for four compounds over a wide range of concentrations. The proposed HPLC-ELSD method provides a useful alternative for the analysis and quality control of multiple bioactive components from the plants in *Dioscorea* L., which could facilitate development for new natural resources of steroidal saponin.

References

Liu JQ, Wang CF, Qiu MH, Hu WX, 2010. Sterodial saponins from flowers of *Hosta plantaginea* and their antitumor activities. *Chin Tradit Herb Drugs* 41(4): 520-526.

Liu ZB, Wang TJ, Lu ZQ, 2008. Simultaneous determination dioscin and protoneodioscin in *Dioscorea nipponica* by HPLC. *Chin Tradit Herb Drugs* 39: 774-775.

Liu ZB, Wang TJ, Wen QJ, 2006. Determination of contents of protoneodioscin in *Dioscorea* L. by RP- HPLC. *Chin Tradit Herb Drugs* 37: 1097-1099.

Mo KF, 2003. Determination of contents of diosgenin in *Dioscorea spongiosas* and *D. hypoglauca. Chin Tradit Herb Drugs* 34: 470-471.

Mo KF, Yang H, 2003. Determination of contents of diosgenin in *Dioscorea spongiosas* and *D. hypoglauca* by TLCs. *China New Med* 11: 35-36.

Pharmacopeia Committee of P. R. China, 2010. *Pharmacopoeia of People's Republic of China*. China Medical Science and Technology Press: Beijing.

Qin LY, Jia LY, Sun QS, 2007. Determination of contents of dioscin and gracillin in *Dioscorea nipponica* Makino by RP-HPLC. *J Shenyang Pharm Univ* 24: 628-630.

Tang SR, Yang RT, Pan FS, 2007. Steroidal saponin and steroidal sapogenin in Chinese *Dioscorea* L. *J Plant Resour Environ* 16: 64-72.

Wang Y, Liu Y, Yin J, 2007. Progress in studies of *Rhizoma Dioscoreae Septemlobae*. *J Shenyang Pharm Univ* 24: 374-379.

Yin J, Kouda K, Tezuka Y, 2003. Steroidal glycosides from the rhizomes of *Dioscorea spongiosa*. *J Nat Prod* 66: 646-650.

Yin J, Kouda K, Tezuka Y, 2004a. Anti-osteoporotic activity of the water extract of *Dioscorea spongiosa*. *Biol Pharm Bull* 27: 583-586.

Yin J, Kouda K, Tezuka Y, 2004b. New diarylheptanoids from the rhizomes of *Dioscorea spongiosa* and their anti-osteoporotic activity. *Planta Med* 70: 54-58.

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The extract I (IC_{50}=0.079 06 mg/mL) and compound **1** (IC_{50}=0.079 06 mg/mL) showed similar inhibitory effects on α-glucosidase as acarbose (IC_{50}=0.073 37 mg/mL) which is widely used in clinic for the treatment of diabetes, while compounds **2** (IC_{50}=0.879 80 mg/mL) had less activity.

Conclusion

(2*R*,3*R*,5*R*)-2-(hydroxymethyl) piperidine-3,5-diol (**2**) is a new alkaloid isolated from *Bombycis Feculae*, and the inhibitory activity of the extract of *Bombycis Faceces*, compounds **1** and **2**, against α-glucosidase has been first reported.

References

Asano N, Tomioka E, Kizu H, Matsui K, 1994. Sugars with nitrogen in the ring isolated from the leaves of *Morus bombycis*. *Carbohydr Res* 253: 235-245.

Chen ZY, Xiao GS, Chen WD, 2003. Determination of 1-deoxy-nojirimycin in silkworm or silkworm powder by ion chromatograph. *China Sericult* 24: 30-31.

Chen ZY, Xiao GS, Chen WD, 2004. Determination of 1-deoxy-nojirimycin in yellow blood silkworm by ion chromatograph. *Food Sci* 25: 150-151.

Jiangsu New Medical College, 1995. *Glossary of Chinese Herbal Medicines*. Science and Technology Press: Shanghai, 1830-1831.

Johnson CR, Golebiowski A, Braun MP, Sundram H, 1994. Chemoenzymatic synthesis of 1,3-dideoxynojirimycin. *Tetrahedron Lett* 35: 1833-1834.

Ostrowski J, Altenbach HJ, Wischnat R, Brauer DJ, 2003. An aza analogue of iso-levoglucosenone: Synthesis and application of a new building block for imino sugars. *Org Chem* 6: 1104-1110.

Pierre C, Roland R, Tremblay, 1978. P-nitrophenol-α-gluco-pyramoside as substrate for measurement of maltase activity in human semen. *J Clin Chem* 24: 208-211.

Ye ZY, Su J, 1990. Experimental study on medicinal pillow of silkworm. *Bull Sericult* 21: 68-70.