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Original Article

Chemical identification and quantification of Hu-Gu capsule by UHPLC–Q-TOF-MS and HPLC-DAD

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ABSTRACT

Hu-Gu capsule is a traditional Chinese medical formula for preventing glucocorticoid-induced osteoporosis. In this work, an ultra-high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry was developed for chemical identification of the phytochemical compositions in positive ion mode. A total of 41 compounds were detected in Hu-Gu capsule, and 25 compounds were characterized and identified by retention time, accurate mass within 5 ppm error and characteristic fragment ions. Among of them, four major flavonoids were further quantified by high performance liquid chromatography diode array detection in the four batches. The method was validated in terms of calibration curve regression coefficient ($r^2 > 0.9994$), repeatability (RSD < 3.5%), intra- and inter-day precision (RSD < 1.1%), recovery (93.0–106.4%), limit of detection (0.1 μg/ml), and limit of quantification (1 μg/ml). The research could provide the analysis of chemical composition and be helpful to control the quality of HGC.

Introduction

Hu-Gu capsule (HGC) has been used as a traditional medicine in China for preventing glucocorticoid-induced osteoporosis (GIO). The formula is composed of ten Chinese herbs as described in Box 1. According to Chinese National Drug Quality Standard (volume 82), the quality control of HGC mainly confines to only an icariin (≥1.15 mg/0.45 g, per capsule) by high performance liquid chromatography method (The State Commission of Chinese Pharmacopoeia, 2010), which is far from meeting the further depth research of HGC on the pharmacology. To our best knowledge, the global chemical profile of HGC has not been reported, and few reports are available on its quality control (Song et al., 2012). Therefore, it is crucial to develop a qualitative and quantitative method for elucidating the systematic chemical contour of HGC. Nowadays, UHPLC–Q-TOF-MS has become one of dominant tools to investigate the complex constituents of herbal formulas. Unlike single quadruple, ion trap or tandem mass spectrometers, a TOF analyzer has the high resolution, high sensitivity and high accuracy and advantage to provide the excellent mass accuracy for determination of elemental composition and high mass resolution for the difficulty chromatographic separation (Liu et al., 2016; Xiang et al., 2015; Ren et al., 2016).

The aim of present study is to develop a comprehensive analysis method for the chemical profile of HGC by UHPLC–Q-TOF-MS. The structures were elucidated on the basis of their accurate mass and characteristic fragmentation behavior of reference substances. Furthermore, four flavonoids in HGC were quantified by HPLC-DAD.

Experimental

Samples, chemicals, and reagents

HGC (batch number: 20160203, 20160501, 20160502, and 20160503) were produced by Guangdong Annol Pharmaceutical Co., Ltd. Epimedin A, epimedin B, epimedin C, and icariin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing China). The purities of the reference compounds were determined to be above 98% by HPLC-DAD analysis. LC/MS-grade acetonitrile was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). HPLC-grade acetic acid (AS1102-002) was purchased from TEDIA, Inc (Fairfield, USA). Deionized water was purified by Millipore purification system (Millipore, MA, USA).

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Box 1: Composition of Hu-Gu capsule.

Drug Latin name	Species name	Chinese name	Rate
Polygoni Multiflori Radix Praeparata	*Reynoutria multiflora* (Thunb.) Moldenke, Polygonaceae	制何首乌	1.67
Epimedium brevicornu Maxim	*Epimedium brevicornu* Maxim, Berberidaceae	淫羊藿	1.33
Rehmanniae Radix Praeparata	*Rehmannia glutinosa* (Gaertn.) DC., Plantaginaceae	熟地黄	1.67
Testudinis Carapax et Plastrum	*Chinemys reevesii* (Gray), Emydidae	龟甲	1
Morindae Officinalis Radix	*Morinda officinalis* F.C.How, Rubiaceae	巴戟天	1.33
Eucommiae Cortex	*Eucommia ulmoides* Oliv., Eucommiaceae	杜仲	1.33
Dipsaci Radix	*Dipsacus asper* Wall. ex C.B. Clarke, Caprifloiaceae	续断	1.33
Drynariae Rhizoma	*Drynaria roosii* Nakaike, Polypodiaceae	骨碎补	1.33
Angelicae Sinensis Radix	*Angelica sinensis* (Oliv.) Diels, Apiaceae	丹当	1
Dioscoreae Rhizoma	*Dioscorea oppositifolia* L., Dioscoriaceae	山药	1.33

Sample preparation

An aliquot (500 mg) of HGC powder was accurately weighed and soaked with 5 ml 80% methanol for 15 min. It was extracted by ultrasonic treatment (140 W, 25 °C) for 30 min. After centrifugation at 14,800 × *g* for 10 min, the supernatant was filtered through a 0.22 μm filter prior to injection into the UHPLC–Q-TOF-MS and HPLC-DAD system.

Analysis of HGC by UHPLC–Q-TOF-MS

Agilent 1290 series Rapid Resolution LC system was coupled to Agilent 6530 Accurate-Mass Quadrupole Time of Flight (Q-TOF) mass spectrometer (Agilent Technologies, CA, USA). It equipped with an ESI interface. The chromatographic separation was performed on TOSOH TSK gel ODS-100Z (4.6 × 150 mm, 3.0 μm) column. The column temperature was maintained 30 °C, the injection volume was 2 μl. The mobile phase consisted of 0.1% aqueous acetic acid (*v*/*v*) (A) and acetonitrile (B). The gradient program was applied as follows: 0–50 min at 10–45% B; 50–60 min at 40–90% B; 60–62 min at 90–10% B; and 62–70 min at 10% B. The outlet of HPLC was split (1:5) and introduced into the ESI source at 4–62 min and into waste at 0–4 min and 62–70 min.

The MS conditions were set as follows: drying gas flow rate 10 l/min; drying gas temperature 350 °C; pressure of nebulizer gas pressure 42 psig; sheath gas temperature 335 °C; sheath gas flow 11 l/min; capillary voltage 3500 V positive ion modes and the mass range from *m/z* 200 to 1000. The MS/MS spectra were acquired with auto MS/MS mode at the scan rate of 2 spectra/s. The data was acquired with Mass Hunter Acquisition B.06.00 and analyzed by means of Mass Hunter Qualitative Software, Version B.06.00 (Agilent Technologies, CA, USA).

Quantification by HPLC-DAD

Quantification was performed on a Shimadzu liquid Chromatography system. The instrument was equipped with two LC-20AT pumps, a SIL-20A auto sampler, a CTO-20AC column oven, a SPD-M20A diode array detector, a CBM-20A communications bus module, and a DGU-20A_3 degasser. LC solution software version 1.24 SP1 was used to control the HPLC system and process the data.

A TOSOH TSK gel ODS-100Z (4.6 × 150 mm, 3 μm) column (Tosoh Bioscience, Japan) was used for the chromatographic separation. The column temperature was maintained 35 °C, the injection volume was 5 μl, and detection wavelength was set at 269 nm. The mobile phases were composed of water (0.2% aqueous acetic acid, A) and acetonitrile (B). The gradient program was applied as follows: 0–50 min at 10–35% B; 50–60 min at 35–95% B; 60–62 min at 95–10% B; and 62–70 min at 10% B. Flow rate was 1 ml/min.

Results and discussion

A total of 41 compounds were detected in HGC, and 25 compounds were tentatively identified and characterized by UHPLC–Q-TOF-MS in the positive mode. They are summarized along with their retention time, theoretical mass, molecular formula, observed mass, MS/MS fragments as shown in Table 1. A total ion chromatogram and extract ion chromatogram of 25 identified compounds are shown in Fig. 1. Tandem mass spectra and possible fragment pathways of 12 typical constituents in positive mode are presented in Fig. S1.

Characterization and identification of prenylated flavonoids

Fifteen prenylated flavonoids were identified in HGC. The prenylated flavonoid glycosides are characteristic constituents of *Epimedium brevicornu*. The anhydroicaritin flavonoids are usually 3-*O*, 7-*O*, and 3, 7-di-*O*-glycosides with a monosaccharide or disaccharide (Wang et al., 2010). The characteristic fragmentation pathways indicated that glucose substituted at the 3-*O* position lost easier than at the 7-*O* position. The ion of *m/z* 369 was formed. The rearrangement of the isopentane group in position C8 led to the formation of a product ion *m/z* 313 (Shevlyakova et al., 2016).

Compounds 29, 32, 33, and 34 were identified by comparison with reference substances. They were identified as epimedin A, epimedin B, epimedin C, and icariin, respectively. The epimedin A, B, C, and icariin indicated the similarities of fragmentation patterns. All of them presented three characteristic fragment ions at *m/z* 531, 369, and 313. The ion of *m/z* 369 was the typical fragment for anhydroicaritin aglycone. The ion at *m/z* 313 was formed by further loss of C_4H_7 (56 Da) from the ion of *m/z* 369. For the prenylated flavonoids, the glucose substituted at the 3-*O* position lost easier than at the 7-*O* position according to the characteristic fragmentation pathways. The MS^2 spectrum gave the base peak at *m/z* 531, which corresponded to anhydroicaritin aglycone bearing *O*-linked monosaccharide substituent (Ding et al., 2011).

Compounds 31 (t_R = 32.70 min), 36 (t_R = 39.40 min), 38 (t_R = 52.02 min), and 41 (t_R = 54.39 min), $[M+H]^+$ ions indicated at *m/z* 839.2971, 677.2455, 531.1874, and 515.1933, respectively. The MS^2 spectrum gave the same characteristic fragment ions at *m/z* 369 and 313. Compounds 31, 36, 38, and 41 possess an anhydroicaritin aglycone skeleton. Compound 31 gave the base peak at *m/z* 531, due to the loss of both glucose and rhamnose moieties (308 Da). The ions were cleavaged at *m/z* 677 $[M–Glu+H]^+$, 531 $[M–Glu–Rha+H]^+$, and 369 $[M–2Glu–Rha+H]^+$, respectively. Compound 31 was tentatively identified as epimedin A1. Compound 36 yielded a major fragment ion at *m/z* 369, due to the loss of both glucose and rhamnose moieties (308 Da). The ions were fragmented at *m/z* 531 $[M–Rha+H]^+$ and 313 $[M–Glu–Rha–C_4H_7+H]^+$, respectively. It was tentatively identified as sagittatoside A (Guan

Table 1
Identification of the chemical constituents from HGC by UHPLC–Q-TOF-MS.

Peak No.	t_R (min)	Theoretical mass	$[M+H]^+$ or $[M+Na]^+$	Error (ppm)	Observed mass	λ_{max} (nm)	MS/MS fragments (relative ion intensity, %)	Identification compounds
1	5.51	365.0867	$C_{17}H_{16}O_9$	−3.00	365.0878	221, 293	288.0345 (43), 203.0509 (100), 180.0639 (50), 136.0570 (30), 104.9880 (69)	Unknown
2	5.74	379.0660	$C_{17}H_{14}O_{10}$	0.19	379.0659	231, 293	339.1278 (14), 295.0779 (15), 233.0214 (100), 197.5969 (10), 169.0289 (17), 147.0468 (63)	Unknown
3	5.82	399.1286	$C_{18}H_{22}O_{10}$	0.18	399.1285	–	255.0827 (58), 237.0741 (100), 203.0459 (23), 167.0317 (31), 149.0169 (14)	Unknown
4	6.12	441.1391	$C_{20}H_{24}O_{11}$	1.45	441.1385	196, 255	205.0092 (100)	Unknown
5	7.07	515.2639	$C_{29}H_{38}O_8$	−2.05	515.2650	–	269.1270 (100), 210.0535 (67), 182.0557 (16)	Unknown
6	7.36	359.1489	$C_{20}H_{22}O_6$	2.55	359.1480	236, 280	191.0669 (100)	Unknown
7	7.83	355.1024	$C_{16}H_{18}O_9$	−4.07	355.1038	240, 328	163.0393 (100), 145.0297 (17), 135.0436 (6), 117.028 (7)	Chlorogenic acid
8	8.06	355.1024	$C_{16}H_{18}O_9$	−4.07	355.1038	242, 328	163.0393 (100), 145.0314 (14), 135.0434 (9), 117.0393 (3)	Cryptochlorogenic acid
9	9.21	509.1290	$C_{23}H_{24}O_{13}$	−0.06	509.1290	252, 282	509.1291 (100), 363.0718 (13), 331.1036 (46), 201.0154 (11)	Unknown
10	9.55	413.1442	$C_{19}H_{24}O_{10}$	1.51	413.1436	245	413.1426 (100), 251.0932 (11), 219.0611 (20)	Cnidioside B methyl ester
11	9.72	739.2444	$C_{34}H_{42}O_{18}$	1.61	739.2432	–	381.1175 (100), 219.0630 (6), 149.0207 (3)	Unknown
12	11.98	342.1700	$C_{20}H_{23}NO_4$	−3.86	342.1713	–	342.1706 (28), 297.1140 (82), 282.0874 (22), 265.0861 (100), 237.0942 (24)	Magnoflorine
13	13.77	413.2170	$C_{21}H_{32}O_8$	0.96	413.2166	241	413.2163 (100), 203.0536 (15)	Unknown
14	16.85	547.2174	$C_{28}H_{34}O_{11}$	1.99	547.2163	–	547.2151 (100), 385.1600 (18)	3-Hydroxyicariine-*O*-glucoside
15	17.09	619.1633	$C_{27}H_{31}O_{15}Na$	−4.46	619.1661	230, 285	619.1640 (100), 473.1125 (3), 331.1005 (12)	Neoeriocitrin
16	17.56	363.1438	$C_{19}H_{22}O_7$	1.19	363.1434	243, 322	363.1366 (100), 191.0420 (35), 117.0669 (45)	Unknown
17	18.13	407.1337	$C_{20}H_{22}O_9$	−4.78	407.1356	230, 320	245.0818 (100), 227.0715 (32), 199.0767 (69), 181.0603 (10), 151.0392 (46), 125.0244 (99), 121.0637 (37), 107.0484 (17)	Stilbene glucoside
18	18.69	355.1751	$C_{18}H_{26}O_7$	1.78	355.1745	–	355.1726 (100), 131.0513 (7)	Unknown
19	19.36	518.2146	$C_{27}H_{33}O_{10}$	1.25	518.2140	–	518.2109 (98), 356.1590 (100)	Unknown
20	19.87	435.2589	$C_{21}H_{38}O_9$	1.98	435.2580	–	435.2529 (100), 349.1808 (34), 321.1940 (9), 293.1556 (9)	Unknown

Table 1 (*Continued*)

Peak No.	t_R (min)	Theoretical mass	$[M+H]^+$ or $[M+Na]^+$	Error (ppm)	Observed mass	λ_{max} (nm)	MS/MS fragments (relative ion intensity, %)	Identification compounds
21	21.22	581.1865	$C_{27}H_{32}O_{14}$	−2.27	581.1878	229, 284	339.0889 (17), 315.0855 (16), 297.0741 (20), 273.0772 (100), 195.0279 (55), 153.0171 (13), 129.0547 (22)	Naringin
22	22.12	769.2550	$C_{35}H_{44}O_{19}$	1.24	769.2540	243, 328	769.2556 (100), 607.2022 (5), 395.1308 (5)	Unknown
23	23.09	247.0965	$C_{14}H_{14}O_4$	2.79	247.0958	278	187.8379 (100)	Diallyl phthalate
24	25.48	825.2812	$C_{38}H_{48}O_{20}$	0.93	825.2804	–	663.2378 (8), 517.1745 (100), 355.1176 (58)	Diphylloside A
25	27.42	489.2119	$C_{26}H_{32}O_9$	0.84	489.2115	–	489.2111 (23), 309.1462 (100), 281.1513 (11)	Unknown
26	27.46	663.2283	$C_{32}H_{38}O_{15}$	−2.35	663.2299	–	517.1756 (23), 355.1194 (100), 299.0526 (6)	Epimedoside A
27	28.51	693.2389	$C_{33}H_{40}O_{16}$	−1.86	693.2402	–	547.1816 (38), 385.1317 (100), 367.1011 (11), 329.0609 (18)	3-Hydroxyicariine-*O*-glucose-rhamnose
28	31.29	431.1337	$C_{22}H_{22}O_9$	1.53	431.1330	–	431.1257 (100), 269.0802 (57)	Ononin
29	31.81	839.2968	$C_{39}H_{50}O_{20}$	−1.29	839.2979	270	677.2451 (7), 531.1903 (100), 369.1363 (45), 313.0665 (10)	Epimedin A
30	32.49	455.0973	$C_{23}H_{18}O_{10}$	2.14	455.0963	–	455.1004 (29), 293.0420 (100), 185.0394 (59)	Unknown
31	32.70	839.2968	$C_{39}H_{50}O_{20}$	−0.33	839.2971	–	677.2454 (6), 531.1862 (100), 369.1313 (55), 313.0797 (4)	Epimedin A_1
32	33.64	809.2863	$C_{38}H_{48}O_{19}$	−2.41	809.2882	270	677.2454 (8), 531.1878 (100), 369.1329 (52), 313.0665 (7)	Epimedin B
33	34.17	823.3019	$C_{39}H_{50}O_{19}$	−0.24	823.3021	270	677.2418 (12), 531.1863 (100), 369.1338 (56), 313.0691 (5)	Epimedin C
34	35.45	677.2440	$C_{33}H_{40}O_{15}$	−1.78	677.2452	270	531.1874 (26), 369.1336 (100), 313.0702 (17)	Icariin
35	38.64	469.1129	$C_{24}H_{20}O_{10}$	3.25	469.1114	270	469.1089 (47), 307.0555 (100), 185.0364 (67)	Epigallocatechin 3-*O*-caffeoate
36	39.40	677.2440	$C_{33}H_{40}O_{15}$	−2.22	677.2455	–	531.1830 (30), 369.1328 (100), 313.0685 (6)	Sagittatoside A
37	47.17	523.1599	$C_{26}H_{27}O_{10}Na$	−0.63	523.1602	–	377.1013 (100), 318.9111 (9), 169.0395 (8)	Ikarisoside A (Baohuoside II)
38	52.02	531.1861	$C_{27}H_{30}O_{11}$	−2.47	531.1874	–	369.1341 (100), 313.0711 (29)	Icariside I
39	52.45	669.2154	$C_{32}H_{37}O_{14}Na$	−1.53	669.2164	–	391.1117 (26), 301.0896 (100)	Sagittatoside B
40	52.70	683.2310	$C_{33}H_{40}O_{14}Na$	−2.08	683.2324	–	683.2299 (100), 391.1118 (41), 315.1050 (92)	2″-*O*-rhamnosyl icariside II
41	54.39	515.1912	$C_{27}H_{30}O_{10}$	−4.14	515.1933	–	369.1338 (100), 313.0711 (20)	Icariside II (Baohuoside I)

–, UV spectra have not been observed due to low intensity.

et al., 2011). Compound 38 gave the base peak at *m/z* 369, due to the loss of a glucose moiety (162 Da). The ion of *m/z* 369 was the characteristic fragment for anhydroicaritin aglycone. The ion at *m/z* 313 was formed by the loss of C_4H_7 (56 Da). It was tentatively identified as icariside I (Zhao et al., 2008). Compound 41 yielded two fragment ions at *m/z* 369 $[M–Rha+H]^+$ and 313 $[M–Rha–C_4H_7+H]^+$. Compound 41 was tentatively identified as icariside II or named baohuoside I (Zhao et al., 2008).

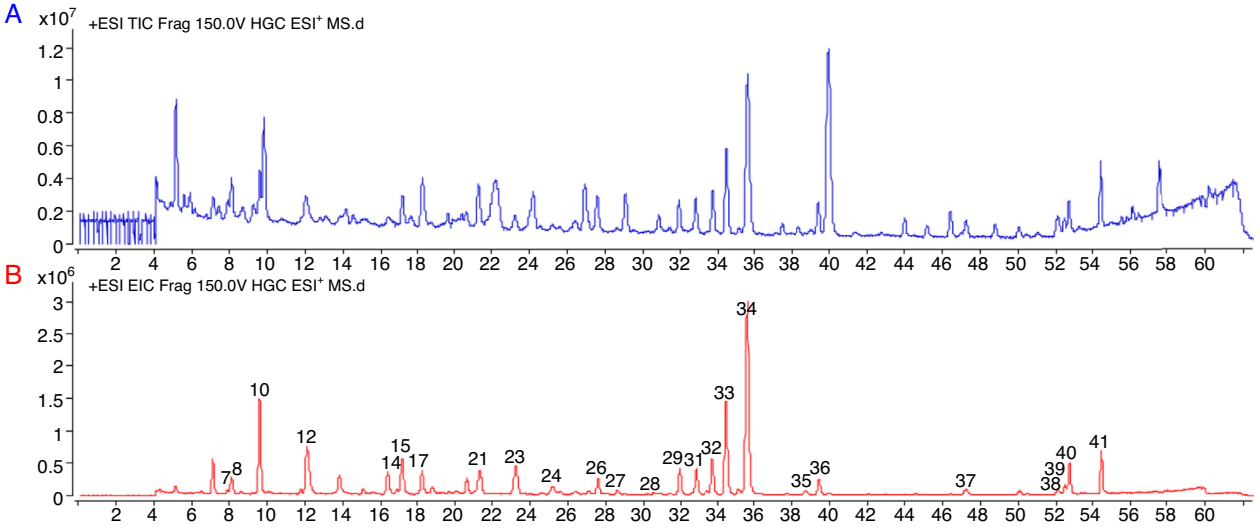

Fig. 1. The total ion chromatogram of HGC (A) and extracted ion chromatogram of 25 identified compounds (B).

Compounds 14 and 27 (t_R = 16.85 and 28.51 min), $[M+H]^+$ pseudomolecular ions presented at *m/z* 547.2163 and 693.2402, which corresponded to the molecular formula of $C_{28}H_{34}O_{11}$ and $C_{33}H_{40}O_{16}$, respectively. In the MS^2 spectra the fragments were at *m/z* 385, which was attributed to the loss of a glucose moiety (162 Da) and both glucose and rhamnose moieties (308 Da). Compound 14 was tentatively identified as 3-hydroxyicariine-*O*-glucoside (Zhao et al., 2008). For compound 27, the fragment ions were at *m/z* 547 $[M–Rha+H]^+$ and *m/z* 367 $[M–Rha–Glu–H_2O+H]^+$. It was tentatively identified as 3-hydroxyicariine-*O*-glucose-rhamnose (Zhao et al., 2008).

Two compounds 24 (t_R = 25.48 min) and 26 (t_R = 27.46 min) indicated $[M+H]^+$ ions were at *m/z* 825.2804 and 663.2299. The MS^2 spectrum of compound 24 gave the fragment ions at *m/z* 663 $[M–Glu+H]^+$ (8% of base peak), *m/z* 517 $[M–Glu–Rha+H]^+$ as base peak, and *m/z* 355 $[M–Glu–Rha–Glu+H]^+$ (58% of base peak). Compound 24 was tentatively identified as diphylloside A in agreement with the literature (Guan et al., 2011). In the MS^2 spectra of compound 26, the fragment ion at *m/z* 517 was attributed to the loss of a rhamnose moiety (146 Da). The base peak was at *m/z* 355, due to the loss of both rhamnose and glucose moieties. It was tentatively identified as epimedoside A.

Compound 37, t_R = 47.17 min, $[M+Na]^+$ ion was at *m/z* 523.1602. The MS^2 spectrum gave the base peak at *m/z* 377, due to the loss of a rhamnose moiety (146 Da). It was tentatively identified as ikarisoside A or named baohuoside II (Guan et al., 2011).

Compounds 39 (t_R = 52.45 min) and 40 (t_R = 52.70 min) exhibited $[M+Na]^+$ ions at *m/z* 669.2164 and 683.2324. In the MS^2 spectrum, the same fragment ion at *m/z* 391 indicated the loss of both rhamnose and xylose moieties (278 Da) and two rhamnose moieties (292 Da), respectively. They were tentatively identified as sagittatoside B and 2″-O-rhamnosyl icariside II (Guan et al., 2011).

Other chemical components

Compounds 7 and 8 (t_R = 7.83 min and 8.06 min) showed $[M+H]^+$ ion at *m/z* 355.1038, which corresponded to the molecular formula of $C_{16}H_{18}O_9$. The MS^2 spectrum gave a base peak at *m/z* 163 [caffeic acid–H_2O+H]$^+$, corresponding to the loss of quinic acid moiety (192 Da). The compounds 7 and 8 indicate the same elemental composition. The MS^2 spectra demonstrate the similar fragmentation and intensity. However, the difference in retention time is likely to identify the two peaks due to linkage. Generally, cryptochlorogenic acid has stronger retention on the C18 column than chlorogenic acid by comparison with the published data (Ding et al., 2011; Zhao et al., 2008). The compound 7 and 8 was tentatively identified as chlorogenic acid and cryptochlorogenic acid. They might be from *Eucommiae Cortex* and *Epimedium brevicornu* Maxim (Zhu et al., 2016; Zhao et al., 2008).

Compound 10 (t_R = 9.55 min), $[M+H]^+$ ion presented at *m/z* 413.1436, which corresponded to the molecular formula of $C_{19}H_{24}O_{10}$. The fragment ion at *m/z* 251 indicated the loss of a glucose moiety. Compound 10 was tentatively identified as cnidioside B methyl ester.

Compound 12 (t_R = 11.98 min), $[M+H]^+$ ion indicated at *m/z* 342.1713, which corresponded to the molecular formula of $C_{20}H_{23}NO_4$. The fragment ions at *m/z* 297 and 265 indicated the loss of –N $(CH_3)_2$ and $–OCH_3$. Compound 12 was tentatively identified as magnoflorine from *Epimedium brevicornu* (Naseer et al., 2015).

Compound 15 (t_R = 17.09 min), $[M+Na]^+$ ion showed at *m/z* 619.1661, which corresponded to the molecular formula of $C_{27}H_{31}O_{15}Na$. The fragment ion at *m/z* 473 indicated the elimination of a rhamnose residue (146 Da) from the precursor ion. It was tentatively identified as neoeriocitrin (Liu et al., 2012).

Compound 17 (t_R = 18.13 min), $[M+H]^+$ ion presented at *m/z* 407.1356 with identical molecular formula $C_{20}H_{22}O_9$. The elemental composition was obtained with an error of less than 5 ppm. The MS^2 spectrum of ion at *m/z* 407 presented the successive fragments of *m/z* 245, 227, 199, 181, 151, 125, 121, and 107. Hence, it was tentatively identified as stilbene glucoside from *Polygoni Multiflori Radix Praeparata* by comparison to the literature (Qiu et al., 2013).

Compound 21, t_R = 21.22 min, $[M+H]^+$ ion at *m/z* 581.1878 gave the molecular formula of $C_{27}H_{32}O_{14}$. The MS^2 spectrum gave the fragment ions at *m/z* 339, 315, 297, 273, 195, 153, and 129. The aglycone ion at *m/z* 273 indicated the consecutive loss of rhamnose and glucose fragment (308 Da). The ion at *m/z* 153 was yielded from retro Diels–Alder fragmentation pattern of the *C*-ring. The neutral loss of 120 Da was attributed to the characteristic ion $[M–C_4H_8O_4+H]^+$. Compound 21 from *Drynariae Rhizoma* was tentatively identified as naringin (Li et al., 2010; Wu et al., 2015).

Compound 23, t_R = 23.09 min, $[M+H]^+$ ion at *m/z* 247.0958 gave the molecular formula of $C_{14}H_{14}O_4$. The prominent fragment ion in the MS/MS spectrum was at *m/z* 187 $[M–C_3H_5O+H]^+$. It was tentatively identified as diallyl phthalate. The compound may be from a contamination of the column or the solvents used in the extraction.

Compound 28, t_R = 31.29 min, $[M+H]^+$ ion exhibited at *m/z* 431.1330. In the MS^2 spectra, the fragment ion was at *m/z* 269,

which was attributed to the loss of a glucose moiety (162 Da). It was tentatively identified as ononin from *Epimedium brevicornu* (Luo et al., 2014).

Compound 35, t_R = 38.64 min, $[M+H]^+$ ion displayed at *m/z* 469.1114. In the MS^2 spectra, the fragment ion was at *m/z* 307, which was attributed to the loss of a caffeic acid group (162 Da). It was tentatively identified as epigallocatechin-*O*-caffeoate.

Calibration curves, limits of detection (LOD) and quantification (LOQ)

For HPLC-DAD quantification, four standard substances were accurately weighed and dissolved in 80% methanol to make the concentration of 1000 μg/ml for epimedin A, epimedin B, epimedin C, and icariin. Mix stock solution was diluted in the 10–500 μg/ml concentration range. The six concentrations for the construction of calibration curves were analyzed in triplicate. The calibration curves were constructed by plotting the peak areas versus the concentrations. Peak areas (x) of serial working solutions were plotted against the corresponding concentrations (y, μg/ml). All the calibration curves showed good linearity with correlation coefficients (r^2) no less than 0.9994. Limit of detection (LOD) and limit of quantification (LOQ) were determined by serial dilution of standard solution until the signal to noise ratio (S/N) for each reference substance got to 3 and 10, respectively. The calibration curves, correlation coefficients (r^2), linear ranges, LOD, and LOQ are presented in Table S1.

Precision, recovery, repeatability, and stability

The standard working solutions used for the intra-day and inter-day precision were the same as those used in the calibration curve experiment. The intra- and inter-day precisions were determined by the relative standard deviation (RSD < 1.1%). The recovery was carried out by adding a known amount of standard substances into HGC (sample 20160502). The spiked samples were extracted using the procedure described for sample preparation. The method recovery was calculated using the equations below:

$$\text{Recovery\%} = \frac{C_{\text{sample+standard}} - C_{\text{sample}}}{C_{\text{standard}}} \times 100.$$

The recovery for each compound at different concentrations ranged from 93.0% to 106.4%. The HGC (sample 20160502) was applied to determine the repeatability and stability. The sample was prepared according to the procedure described as sample preparation. It was analyzed for six replicates in a day for the repeatability. The repeatability of RSD was less than 3.5%. The sample was stored at 25 °C and analyzed at 0, 2, 4, 6, 12 and 24 h for stability test. The stabilities of RSD were less than 0.68%. The precision, recovery, repeatability, and stability are indicated in Table S2. The method was validated according to the guidance for International Council for Harmonisation guidelines Q2 (R1).

Sample analysis

The proposed HPLC-DAD has been used to determine the four major flavonoids in HGC. The quantitative results indicated the contents of epimedin A, epimedin B, epimedin C, and icariin per capsule in four batches of samples as shown in Table S3. The contents of four major compounds in HGC were relatively consistent among four batches. According to Chinese National Drug Quality Standard, the content of icariin should be no less than 1.15 mg/450 mg or per capsule. Our results showed that the four batches complied with this quality standard.

Conclusion

The UHPLC–Q-TOF-MS and HPLC-DAD methods were applied to qualitative and quantitative for the quality control of HGC. Compared with previous investigations, a total of 41 compounds were detected in HGC. Among of them, 25 compounds including fifteen prenylated flavonoids and ten other chemical components were identified on the basis of diagnostic mass data and fragment pattern by UHPLC–Q-TOF-MS in positive mode. The HPLC-DAD method was applied to determine in the four batches of HGC. Prenylated flavonoids are revealed to be the main bioactive ingredients of HGC, which might play important roles in the biological and pharmacological effects. The four potential bioactive compounds in HGC were determined by the developed HPLC-DAD method. The investigation was help for the quality evaluation of HGC.

Authorship

QR designed and performed the experiments; S-SLong analyzed the data. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2017.06.002.

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