Four New Andrographolide Metabolites in Human Urine

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Andrographolide is one of principal components of a famous traditional Chinese herbal medicine *Andrographis paniculate* (BURM) NEES. Four new metabolites of andrographolide were isolated from human urine. All of them were characterized as sulfate and one of them also as a cysteine S-conjugate. The structures were determined to be andrographolide-3-*O*-sulfate (M-1), isoandrographolide-3-*O*-sulfate (M-2), 14-deoxyandrographolide-3-*O*-sulfate (M-3), 14-deoxy-12-(cysteine-S-yl)-andrographolide-3-*O*-sulfate (M-4), respectively, based on chemical evidence and spectroscopic analyses.

Key words andrographolide; metabolites in human urine; sulfate; cysteine S-conjugate

Andrographolide is one of principal constituents of a famous traditional Chinese herbal medicine *Andrographis paniculate* (BURM) NEES. Its chemical structure is 2(3*H*)-furanone, 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro-4-hydroxy. Andrographolide has many bioactivities, such as antiinflammatory,[1—3] anti-allergic,[4,5] anti-platelet aggregation,[6] hepatoprotective[7,8] and anti-human immunodeficiency virus (HIV) activities.[9,10] And this compound has been widely used in clinic for the treatment of fever, cold, inflammation, diarrhea and other infectious diseases. The pharmacokinetic studies showed that andrographolide was quickly absorbed and intensely metabolized in rats and humans.[11] One of the metabolites of it after oral administration in rats was determined as 14-deoxy-12(*R*)-sulfoandrographolide, which was found to be identical to an antiinflammatory drug (Lianbizhi) currently being used in clinic as injection in China.[12,13] Therefore, the metabolites of andrographolide in human urine were further investigated and four sulfate conjugates were obtained. The present paper describes the extraction, isolation and identification of these metabolites.

Metabolite 1 (M-1), white amorphous powder, was positive to the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone. The IR spectrum showed the presence of hydroxyl (3421 cm^{-1}) and ester carbonyl (1736, 1670 cm^{-1}) groups in the molecule. The high-resolution FAB-MS showed the quasi-molecular ion $[M-H]^-$ at *m*/*z* 429.1583 (Calcd 429.1588), corresponding to the molecular formula $C_{20}H_{30}O_8S$, which was further supported by the ^1H- and ^{13}C-NMR spectral data. Metabolite 1 was a 80 mass unit (SO_3) higher than that of andrographolide, suggesting it was a sulfate conjugate. The ^{13}C-NMR data of M-1 were similar to those of the parent drug andrographolide, except for the downfield shifts of C-3 by 7.1 ppm and the upfield shifts of C-2 by 3.2 ppm, respectively. In the HMBC spectrum, H-3 (δ 4.13, 1H, overlapped) had peaks correlated with C-19 (δ 63.6) and C-18 (δ 23.1) while H-18 (δ 1.19, 3H, s) correlated with C-3 (δ 88.0), C-19 (δ 63.6), C-5 (δ 56.8) and C-4 (δ 44.4), confirming the sulfate at C-3.

Based on the above data, metabolite 1 was determined to be andrographolide-3-*O*-sulfate. The full assignments of carbon and proton signals are summarized in Table 1.

Metabolite 2 (M-2), white amorphous powder, was positive to the Legal and Kedde reactions. In the IR spectrum, the absorption at 3421 cm^{-1} was the absorption of the hydroxyl group while the absorption at 1747 and 1670 cm^{-1} were the absorption of the ester carbonyl group. In the high-resolution FAB-MS, a quasi-molecular peak $[M-H]^-$ at *m*/*z* 429.1589 (Calcd 429.1588) was observed, and thus the molecular formula of $C_{20}H_{30}O_8S$ was derived. The assignment of this formula was further supported by the ^1H- and ^{13}C-NMR spectral data. Metabolite 2 was a 80 mass unit (SO_3) higher than that of andrographolide, suggesting it was also a sulfate conjugate. The ^1H- and ^{13}C-NMR data of metabolite 2 were very similar to those of metabolite 1 except the following findings. The proton signal of H-11 (δ 2.89, 2H, m) was shifted downfield by 0.31 ppm, the H-12 (δ 6.50, 1H, t, J=6.4 Hz) signal was shifted upfield by 0.34 ppm, and the carbon signal of C-14 (δ 69.8) was shifted downfield by 3.2 ppm. In the NOESY spectrum, the cross peaks were observed between H-12 (δ 6.50, 1H, t, J=6.4 Hz) and H-14 (δ 4.70, 1H, m) in metabolite 2, while they were absent in metabolite 1, which indicated metabolite 2 was the geometric isomer of metabolite 1 at the 12(13) double bond. Metabolite 2 might be derived from a 12-cation intermediate of andrographolide formed through elimination of the hydroxyl group and double bond migration.[14]

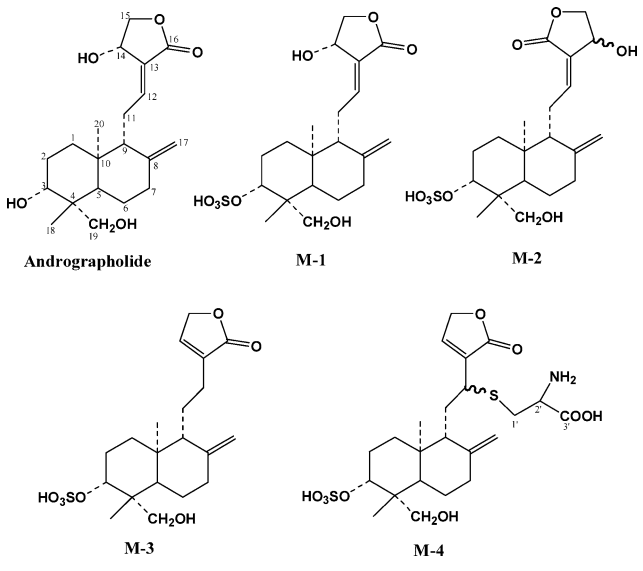

Fig. 1. Structures of Andrographolide and Metabolites 1 to 4

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Table 1. Assignments of Carbon and Proton Signals of Metabolites 1 and 2

No.	Carbon signals			Proton signals[c—e]		
	Andrographolide[a]	M-1[b]	M-2[b]	Andrographolide[a]	M-1[b]	M-2[b]
1	38.1	38.1	38.2	1.83 (H, m) 1.28 (H, m)	1.87 (H, o) 1.33 (H, m)	1.90 (H, o) 1.30 (H, m)
2	29.0	25.8	25.9	1.79 (2H, m)	2.25 (H, m) 1.83 (H, o)	2.26 (H, m) 1.81 (H, o)
3	80.9	88.0	87.9	3.40 (H, m)	4.13 (H, o)	4.10 (H, o)
4	43.6	44.4	44.4			
5	56.3	56.8	57.0	1.32 (H, m)	1.39 (H, o)	1.36 (H, m)
6	25.2	25.6	25.8	1.87 (H, m) 1.36 (H, m)	1.91 (H, o) 1.45 (H, m)	1.85 (H, o) 1.51 (H, m)
7	38.9	38.9	39.1	2.41 (H, m) 2.03 (H, m)	2.42 (H, m) 2.04 (H, m)	2.39 (H, m) 1.98 (H, m)
8	148.7	148.7	149.2			
9	57.4	57.1	57.6	1.92 (H, m)	1.94 (H, o)	1.90 (H, o)
10	39.9	39.9	40.0			
11	25.7	25.6	24.8	2.60 (2H, m)	2.58 (2H, m)	2.89 (2H, m)
12	149.3	149.2	151.2	6.84 (H, dt, J=6.7, 1.8 Hz)	6.84 (H, t, J=6.6 Hz)	6.50 (H, t, J=6.4 Hz)
13	129.8	129.8	129.4			
14	66.6	66.6	69.8	5.00 (H, dd, J=4.2, 1.8 Hz)	5.02 (H, d, J=5.7 Hz)	4.70 (H, m)
15	76.1	76.1	74.9	4.45 (H, dd, J=10.2, 6.1 Hz)	4.46 (H, dd, J=10.2, 6.1 Hz)	4.40(H, dd, J=9.9, 6.1 Hz)
				4.14 (H, dd, J=10.2, 2.1 Hz)	4.15 (H, dd, J=10.2, 1.9 Hz)	4.05 (H, dd, J=9.9, 2.8 Hz)
16	172.6	172.6	171.5			
17	109.2	109.1	108.5	4.87 (H, d, J=1.8 Hz)	4.88 (H, o) 4.66 (H, s)	4.85 (H, o) 4.49 (H, s)
				4.65 (H, d, J=1.1 Hz)		
18	23.3	23.1	23.2	1.20 (3H, s)	1.19 (3H, s)	1.18 (3H, s)
19	64.9	63.6	63.8	4.10 (H, d, J=11.0 Hz)	3.92 (H, d, J=11.8 Hz)	3.92 (H, d, J=11.7 Hz)
				3.36 (H, d, J=11.1 Hz)	3.50 (H, d, J=11.8 Hz)	3.47 (H, d, J=11.7 Hz)
20	15.5	15.0	15.1	0.74 (3H, s)	0.76 (3H, s)	0.75 (3H, s)

a) Recorded on BRUKER ARX 300, in CD_3OD. *b*) Recorded on BRUKER ARX 400, in CD_3OD. *c*) The carbon and proton signals were assigned unambiguously on ^{1}H-, ^{13}C-NMR, COSY, NOESY, HMBC and HMQC. *d*) m, multiple split. *e*) o, overlapped peaks.

Based on the above analysis, M-2 was determined to be isoandrographolide-3-*O*-sulfate except for the absolute configuration at C-14. The full assignments of carbon and proton signals were summarized in Table 1.

Metabolite 3 (M-3), white amorphous powder, was positive to the Legal and Kedde reactions. There were hydroxyl absorption at 3445 cm^{-1} and the ester carbonyl at 1751 cm^{-1} in the IR spectrum. In the negative ESI-MS, the molecular ion peak was shown at *m/z* 414. Thus the molecular formula was determined to be $C_{20}H_{30}O_7S$ with combination of the ^{1}H- and ^{13}C-NMR spectral data. Metabolite 3 was a 16 mass unit less than that of metabolite 1, suggesting metabolite 3 might be a deoxygenated sulfate conjugate. ^{13}C-NMR of metabolite 1 and metabolite 3 showed similarities except for the chemical shifts at C-9 and C-11 to C-16, in which the hydroxyl-linked carbon signal at δ 66.6 (C-14) of metabolite 1 disappeared and a new carbon signal of methylene at δ 25.3 (C-12) was observed. This confirmed that metabolite 3 was a deoxygenated sulfate conjugate. In the ^{1}H–^{1}H COSY spectrum, a stepwise coupling was observed from H-9 (δ 1.66, 1H, overlapped) through H-12 (δ 2.38, 1H, overlapped; δ 2.10, 1H, m) mediated by H-11 (δ 1.72, 1H, m; δ 1.66, 1H, overlapped). In the HMBC spectrum, H-14 (δ 7.33, 1H, m) had correlations with C-16 (δ 176.9), C-13 (δ 134.8), C-15 (δ 72.0) and C-12 (δ 25.3) while H-12 (δ 2.38, 1H, overlapped; δ 2.10, 1H, m) correlated with C-16 (δ 176.9), C-14 (δ 147.6) and C-13 (δ 134.8). These data suggested the structure of the lactone side chain moiety. In compassion with metabolite 1, the double bond of metabolite 3 had transferred from the outside to inside of the lactone ring and the hydroxyl group at C-14 of metabolite 1 disappeared in metabolite 3. In the UV spectrum, the maximal absorption of metabolite 3 was at 203 nm, which was different from that of andrographolide at 225. The hypsochromic shift in UV spectrum also supported the change of conjugated system.

Based on chemical and spectroscopic analyses, M-3 was determined to be 14-deoxyandrographolide-3-*O*-sulfate. The full assignments of carbon and proton signals were summarized in Table 2.

Metabolite 4 (M-4), white amorphous power, was positive to the Legal and Kedde reactions. The IR spectrum showed the presence of hydroxyl (3445 cm^{-1}) and ester carbonyl (1740, 1670 cm^{-1}) groups in the molecule. The high-resolution ESI-MS showed the quasi-molecular ion peak $[M+2Na-H]^+$ at *m/z* 578.1492 (Calcd 578.1470). The molecular formula was determined to be $C_{23}H_{35}O_9NS_2$ in combination with the ^{1}H- and ^{13}C-NMR spectral data. The negative ESI mass spectrum of metabolite 4 showed the quasi-molecular ion peak $[M-H]^-$ at *m/z* 532 and the MS/MS spectrum provided two fragment ions at *m/z* 445 ($[M-CH_2=C(NH_2)COOH]^-$) and *m/z* 411 ($[M-\text{cysteine}]^-$), suggesting that the metabolite 4 was a cysteine conjugate.[15] ^{13}C-NMR data of metabolite 4 and metabolite 3 showed close similarity except for C-9, C-11 to C-16, and a group of additional carbon signals at δ 33.4 (C-21), δ 55.6 (C-22) and δ 171.5 (C-23) corresponding to the cysteine moiety.[16] In the ^{1}H–^{1}H COSY spectrum, a stepwise coupling from H-9 (δ 2.24, 1H, overlapped) through H-12 (δ 3.65, 1H, overlapped) mediated by H-11 (δ 1.91, 2H, overlapped) and an independent vicinal coupling from H-14 (δ 7.60, 1H, s) to H-15 (δ 4.90, 2H, overlapped) were observed. In the HMBC spectrum, H-14 (δ 7.60, 1H, s) showed correlations with C-16 (δ 175.1), C-13 (δ 135.3), C-15 (δ 72.1) and C-12 (δ 41.7) while both H-12 (δ 3.65, 1H, overlapped) and H-15 (δ 4.90, 2H, overlapped) correlated with C-16 (δ 175.1), C-14 (δ 149.0) and C-13 (δ 135.3). On the basis of the ^{1}H–^{1}H COSY and HMBC data,

Table 2. Assignments of Carbon and Proton Signals of Metabolites 3 and 4

No.	Carbon signals		Proton signals[b—d]	
	M-3[a]	M-4[a]	M-3[a]	M-4[a]
1	38.1	37.9	1.82 (H, o) 1.21 (H, m)	1.84 (H, o) 1.38 (H, m)
2	25.9	25.8	2.22 (H, m) 1.79 (H, o)	2.24 (H, o) 1.81 (H, o)
3	88.0	87.8	4.07 (H, dd, J=11.8, 4.7 Hz)	4.09 (H, dd, J=11.8, 4.3 Hz)
4	44.4	44.4		
5	57.1	57.0	1.31 (H, m)	1.42 (H, m)
6	25.9	25.8	1.85 (H, o) 1.48 (H, m)	1.89 (H, o) 1.51 (H, m)
7	39.4	39.4	2.42 (H, m) 1.97 (H, m)	2.43 (H, m) 2.03 (H, m)
8	148.8	148.7		
9	57.1	54.5	1.66 (H, o)	2.24 (H, o)
10	40.1	40.0		
11	23.1	29.1	1.72 (H, m), 1.66 (H, o)	1.91 (2H, o)
12	25.3	41.7	2.38 (H, m), 2.10 (H, m)	3.65 (H, o)
13	134.8	135.3		
14	147.6	149.0	7.33 (H, m)	7.60 (H, s)
15	72.0	72.1	4.81 (2H, o)	4.90 (2H, o)
16	176.9	175.1		
17	107.5	108.1	4.87 (H, s) 4.64 (H, s)	4.90 (H, o) 4.64 (H, s)
18	23.2	23.3	1.17 (3H, s)	1.19 (3H, s)
19	63.8	63.8	3.92 (H, d, J=11.8 Hz)	3.90 (H, d, J=11.9 Hz)
			3.43 (H, d, J=11.8 Hz)	3.47 (H, d, J=11.9 Hz)
20	15.1	15.4	0.70 (3H, s)	0.72 (3H, s)
1′		33.4		3.09 (H, dd, J=14.3, 3.4 Hz)
				2.80 (H, dd, J=14.7, 9.3 Hz)
2′		55.6		3.65 (H, o)
3′		171.5		

a) Recorded on BRUKER ARX 400, in CD_3OD. *b*) The carbon and proton signals were assigned unambiguously on ^{1}H-, ^{13}C-NMR, COSY, NOESY, HMBC and HMQC. *c*) m, multiple split. *d*) o, overlapped peaks.

the structure of the side chain moiety could be established except for the C-12 substituted group. The linked position of the cysteine moiety at C-12 through the sulfur atom was determined by the analysis of the HMBC spectrum, in which the signal of H-1′ (δ 2.80, 1H, dd, J=14.7, 9.3 Hz) showed correlations with C-12 (δ 41.7).

From the above analyses, metabolite 4 was determined to be 14-deoxy-12-(cysteine-S-yl)-andrographolide-3-*O*-sulfate except for the absolute configuration at C-12. The full assignments of carbon and proton signals were summarized in Table 2.

Experimental

General Experimental Procedure IR spectra were determined on a SHIMADZU FT/IR-8400 spectrometer in KBr pellets. UV spectra were measured on a Shimadzu UV-2201 spectrometer. ESI-MS spectra were recorded on a Bruker esquire 2000 mass spectrometer. HR-FAB-MS were recorded in on a Bruker second ionization mass spectrometer. NMR spectra were measured on Bruker AV-400 or Bruker ARX-300 spectrometers, using TMS as an internal standard.

Silica gel for column chromatography (200—300 mesh), and silica gel G_{60} and GF_{254} for thin-layer chromatography were products of Qingdao Marine Chemical Factory. Normal-phase and reverse-phase preparatory TLC was performed using products from Merck Company. D101 were purchased from Tianjing and Rensui, respectively. Sephadex® LH-20 and ODS were the products of Pharmacia Company. Preparative HPLC was performed using an C-8 column (C-8, 250×20 mm, Inertsil Pak; detector: UV).

Subjects and Dosing Procedure Eight healthy volunteers aged 21 to 28 years and weighed 50 to 80 kg (all males) participated in this study. Each subject was given orally 3 tablets 3 times per day (50 mg of andrographolide per tablet) for 2 d and the urine was collected between 0 to 72 h.

Extraction and Isolation The urine samples (approximately 50000 ml in total) were concentrated to almost dryness *in vacuo* after collection. The residue was suspended in water and partitioned with ethyl acetate and *n*-butanol for 3 times, respectively. The butanol layer was subjected to D101 and eluted with H_2O/EtOH stepwisely. The 50% EtOH eluate was further subjected to ODS, Sephadex LH-20 and finally purified by preparative HPLC to yield 5.5 mg of metabolite 1, 6.2 mg of metabolite 2 and 3.0 mg of metabolite 3. The water layer was treated in a similar fashion to give 2.8 mg of metabolite 4.

Metabolite 1, white amorphous powder, $C_{20}H_{30}O_8S$. Legal and Kedde reactions: red. UV (MeOH) [nm]: 202, 223. IR (KBr) cm^{-1}: 3421, 2947, 1736, 1670, 1211, 1056, 972, 921. HR-FAB-MS *m/z*: $[M-H]^-$ 429.1583 (Calcd 429.1588).

Metabolite 2, white amorphous powder, $C_{20}H_{30}O_8S$. Legal and Kedde reactions: red. UV (MeOH) [nm]: 202, 223. IR (KBr) cm^{-1}: 3421, 2927, 1747, 1670, 1242, 1056, 972, 925. HR-FAB-MS *m/z*: $[M-H]^-$ 429.1589 (Calcd 429.1588).

Metabolite 3, white amorphous powder, $C_{20}H_{30}O_7S$, Legal and Kedde Reactions: red. UV (MeOH) [nm]: 203. IR (KBr) cm^{-1}: 3445, 2947, 1751, 1647, 1222, 1060, 975, 925. ESI-MS (−) *m/z*: 413 $[M-H]^-$.

Metabolite 4, white amorphous powder, $C_{23}H_{39}O_9NS_2$. Legal and Kedde reactions: red. UV (MeOH) [nm]: 203. IR (KBr) cm^{-1}: 3445, 2947, 1740, 1670, 1265, 1218, 1056, 968. HR-ESI-MS *m/z*: $[M+2Na-H]^+$ 578.1492 (Calcd 578.1470). ESI-MS (−) *m/z*: 532 $[M-H]^-$.

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