CHAPTER 3
RESULTS

3.1 Ganoderic acids A and F contents in Ling Zhi preparations

The HPLC chromatogram obtained from the standard mixture of ganoderic acids A, F and IS is shown in Figure 5. Ganoderic acid A was eluted first from the HPLC system, followed by ganoderic acid F and IS with the retention time of 28.56, 42.64 and 52.99 min, respectively. All peaks were clearly separated and no peak interference was observed at the retention times of ganoderic acids A and F as well as IS. The regression equations, r^2, linear ranges, LOD as well as LOQ of ganoderic acids A and F under the HPLC conditions used in this study are presented in Table 3A. The linear regression of both ganoderic acids A and F exhibited good linearity within the test range. The % CV of intra- and inter-day assay validation for ganoderic acids A and F were all less than 2% (Table 3B), indicating acceptable precision of the developed analytical method.

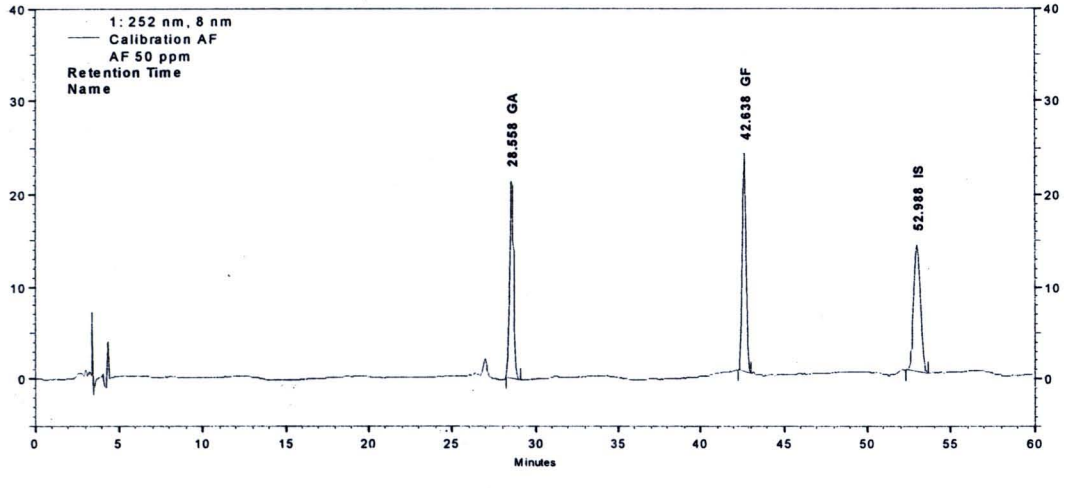


Figure 5 HPLC chromatogram from standard mixture of ganoderic acid A (GA, 50.00 μg/mL), ganoderic acid F (GF, 50.00 μg/mL) and IS (20.00 μg/mL).

Table 3A Regression equations, r^2, linear ranges, LOD and LOQ of ganoderic acids A and F under the HPLC condition used in this study

Compound	Regression equation	r^2	Linear range (μg/mL)	LOD* (μg/mL)	LOQ# (μg/mL)
Ganoderic acid A	y = 20.4114x - 0.1285	1.0000	2.50-200.00	0.73	2.21
Ganoderic acid F	y = 18.0097x + 3.2104	1.0000	2.50-200.00	0.67	2.03

* LOD under the chromatographic conditions was determined based on signal-to-noise ratios (S/N) of 3 : 1

LOQ under the chromatographic conditions was determined based on S/N 10 : 1

Table 3B Intra- and inter-day assay validation of ganoderic acids A and F

Compound	Concentration (μg/mL)	Intra-day precision		Inter-day precision				
				Day 1	Day 2	Day 3	Overall	
		Mean ± SD[1]	% CV	Mean ± SD[1]	Mean ± SD[1]	Mean ± SD[1]	Mean ± SD[2]	% CV
Ganoderic acid A	7.50	7.33 ± 0.05	0.74	7.33 ± 0.12	7.47 ± 0.13	7.33 ± 0.05	7.38 ± 0.08	1.10
	90.00	89.04 ± 0.59	0.66	88.61 ± 0.88	89.47 ± 0.56	89.04 ± 0.59	89.04 ± 0.43	0.48
	180.00	183.36 ± 1.31	0.71	179.73 ± 1.79	180.82 ± 1.54	183.36 ± 1.31	181.30 ± 1.86	1.03
Ganoderic acid F	7.50	7.31 ± 0.07	0.89	7.12 ± 0.10	7.35 ± 0.14	7.31 ± 0.07	7.26 ± 0.12	1.69
	90.00	87.58 ± 0.79	0.90	87.25 ± 0.86	88.18 ± 0.62	87.58 ± 0.79	87.67 ± 0.47	0.54
	180.00	180.34 ± 1.43	0.80	177.29 ± 1.35	178.81 ± 1.87	180.34 ± 1.43	178.81 ± 1.53	0.85

[1] Data represents mean ± SD of the five repetitions of the corresponding concentrations of ganoderic acids analyzed on the specific day

[2] Data represents the overall mean ± SD averaged from the mean values of day 1, 2 and 3

The contents of ganoderic acids A and F in the 19 investigated Ling Zhi preparations including MG2FB and MG2FB-WE are shown in Table 4. Ganoderic acids A and/or F were detected in 16 out of 19 preparations. The ratios of ganoderic acids A to F content in most preparations were approximately 1.10-3.50, indicating that ganoderic acid A was the major compound in comparison to ganoderic acid F, except for NPN in which ganoderic acid F was the major compound. However, only ganoderic acid A was detected in GNO. Among the 16 Ling Zhi preparations in which ganoderic acid(s) existed, the first 3 preparations containing the highest contents of total ganoderic acids were those of 100% Ling Zhi extract namely NPN, MG2FB-WE and DXN-r, respectively. The HPLC chromatograms of 19 Ling Zhi preparations are shown in Figure 6.

It is worth noting that the different dosage forms of 100% crushed Ling Zhi or 100% Ling Zhi fruiting bodies under the same trade name (DHP-c, DHP-t and DHP-s as well as DTG-t and DTG-s) demonstrated the comparable contents of total ganoderic acids, as well as the comparable ratios of ganoderic acids A to F content (i.e., 2.00-2.20 for DHP and 2.30-2.60 for DTG). In contrast, the total ganoderic acids contents of different dosage forms of 100% Ling Zhi extract *versus* 100% Ling Zhi mycelium and sprout extract under the same trade name (DXN-r *versus* DXN-g) were considerably different, since DXN-r possessed the remarkably high ganoderic acids contents whereas neither ganoderic acid A nor F was detected in DXN-g.

Neither ganoderic acid A nor F was detected in 3 out of 19 preparations (GEC, DXN-g and BNR). Among these preparations, the constituent of 2 preparations (GEC and DXN-g) were 100% Ling Zhi extract, whereas the remaining preparation (BNR) was 60% Ling Zhi water extract.

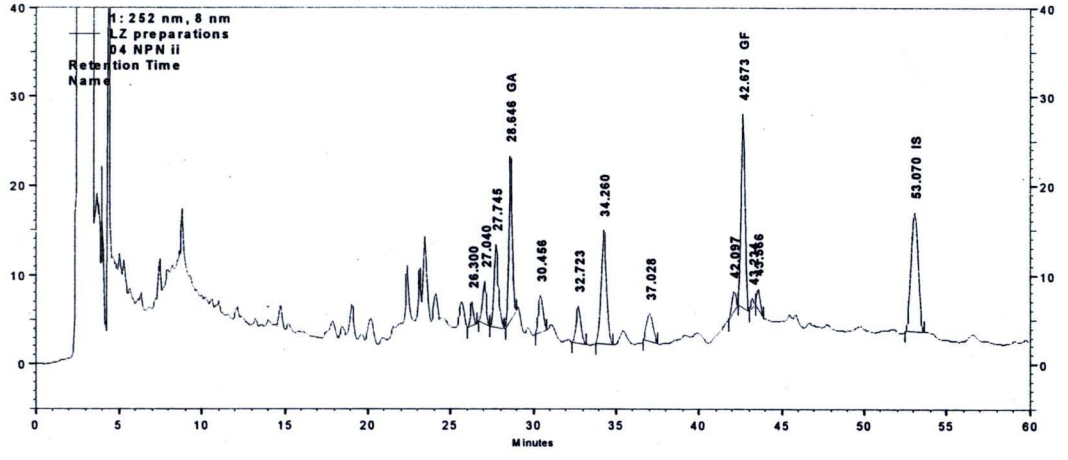
Interestingly, the total contents of ganoderic acids in the 17 commercially available Ling Zhi preparations were not statistically correlated with their prices (Table 4, Figure 7).

Table 4 The contents of ganoderic acids A and F in Ling Zhi preparations investigated in this study[#]

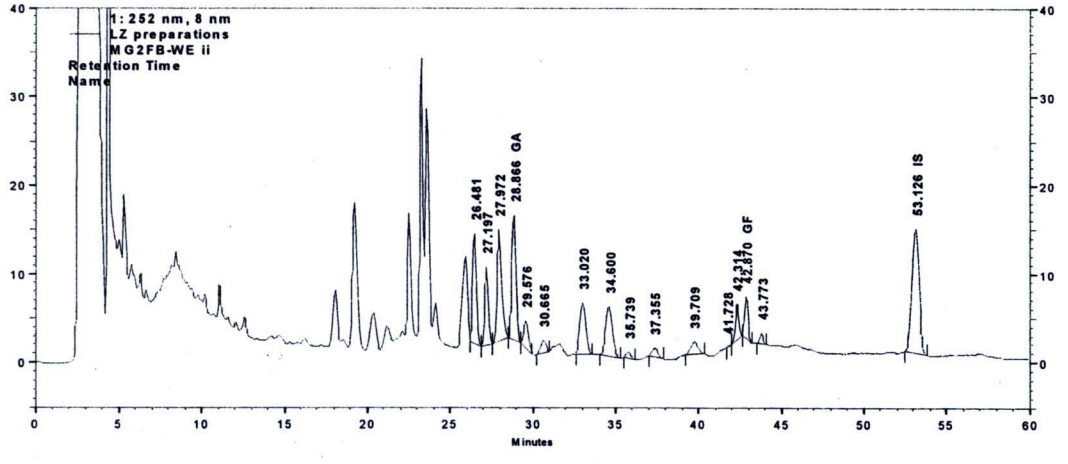
Preparation	Sample No.	Sample code	Constituents	Price (baht/g)	Ganoderic acid A (μg/g)	Ganoderic acid F (μg/g)	Total ganoderic acids[δ] (μg/g)
Powder from capsule or extract	1	NPN	100% Ling Zhi extract	66.67	3,992.88 ± 63.17	4,730.21 ± 84.43	8,723.10 ± 146.53
	2	MG2FB-WE	100% Ling Zhi extract	*	3,100.63 ± 62.11	879.39 ± 84.52	3,980.01 ± 28.34
	3	DXN-r	100% Ling Zhi extract	38.27	1,361.55 ± 11.23	1,264.22 ± 14.84	2,625.77 ± 26.04
	4	DHP-c	100% crushed Ling Zhi	6.67	802.83 ± 5.84	369.51 ± 7.02	1,172.31 ± 7.07
	5	HAV	50% broken spore, 50% Ling Zhi water extract	160.00	791.92 ± 26.35	274.62 ± 8.31	1,066.55 ± 21.32
	6	TYP	100% crushed Ling Zhi	3.75	699.57 ± 39.36	359.71 ± 27.65	1,059.28 ± 65.06
	7	OTH	100% crushed Ling Zhi	14.35	737.51 ± 6.32	301.82 ± 5.76	1,039.33 ± 11.13
	8	DXN-g	100% Ling Zhi extract (mycelium and sprout)	22.96	-	-	-
	9	GEC	100% Ling Zhi extract (mycelium)	18.67	-	-	-
			Median (Range)		802.83 (699.57-3,992.88)	369.51 (274.62-4,730.21)	1,172.31 (1,039.33-8,723.10)
Materials from tea bag or powder from instant tea	10	DHP-t	100% crushed Ling Zhi	1.25	1,175.55 ± 25.88	563.30 ± 13.76	1,738.84 ± 36.89
	11	PTA	100% crushed Ling Zhi	3.33	840.10 ± 23.06	554.89 ± 14.19	1,395.00 ± 36.77
	12	DTG-t	100% crushed Ling Zhi	3.00	794.18 ± 40.23	310.91 ± 14.89	1,105.08 ± 54.58
	13	GNO	20% crushed Ling Zhi, 80% tea powder	6.50	233.80 ± 33.33	-	233.80 ± 33.33
	14	BNR	60% Ling Zhi water extract, 40% sugar	0.26	-	-	-
			Median (Range)		817.14 (233.80-1,175.55)	554.89 (310.91-563.30)	1,250.04 (233.80-1,738.84)
Sliced fruiting bodies	15	DTG-s	100% Ling Zhi fruiting bodies	1.30	1,159.11 ± 12.84	519.96 ± 7.05	1,679.07 ± 11.72
	16	DHP-s	100% Ling Zhi fruiting bodies	2.50	908.38 ± 31.29	459.82 ± 3.94	1,368.20 ± 30.12
	17	JLD	100% Ling Zhi fruiting bodies	3.33	834.26 ± 22.43	282.82 ± 13.34	1,117.08 ± 29.32
	18	MG2FB	100% Ling Zhi fruiting bodies	*	764.43 ± 1.14	240.09 ± 2.80	1,004.52 ± 1.66
	19	MKI	100% Ling Zhi fruiting bodies	10.00	518.40 ± 33.06	468.74 ± 6.91	987.14 ± 29.88
			Median (Range)		834.26 (518.40-1,159.11)	459.82 (240.09-519.96)	1,117.08 (987.14-1,679.07)

[#] Data represents mean ± SD of three repetitions of measurement; [δ] Summative contents of ganoderic acids A and F; * The products kindly provided from Muang Ngai special agricultural project under the patronage of Her Majesty Queen Sirikit, Chiang Mai; (-) means lower than the LOQ

NPN



MG2FB-WE



DXN-r

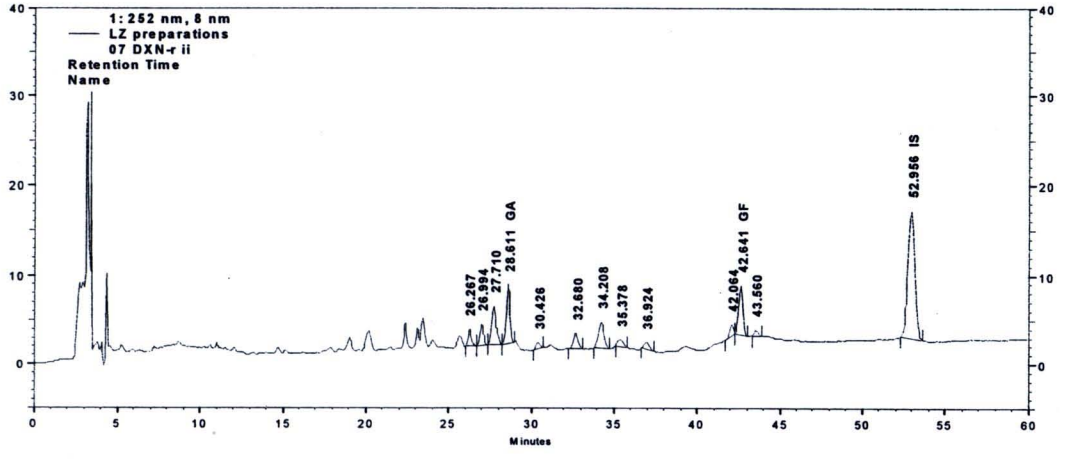
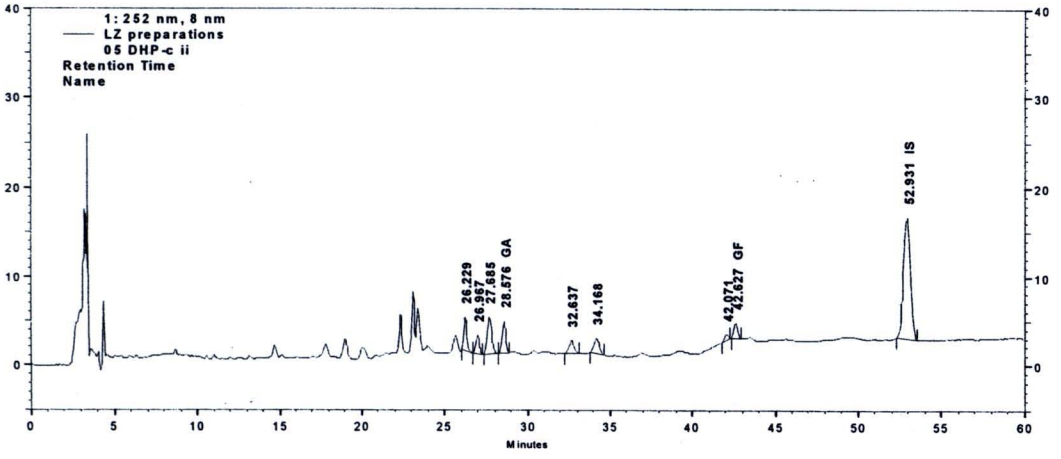
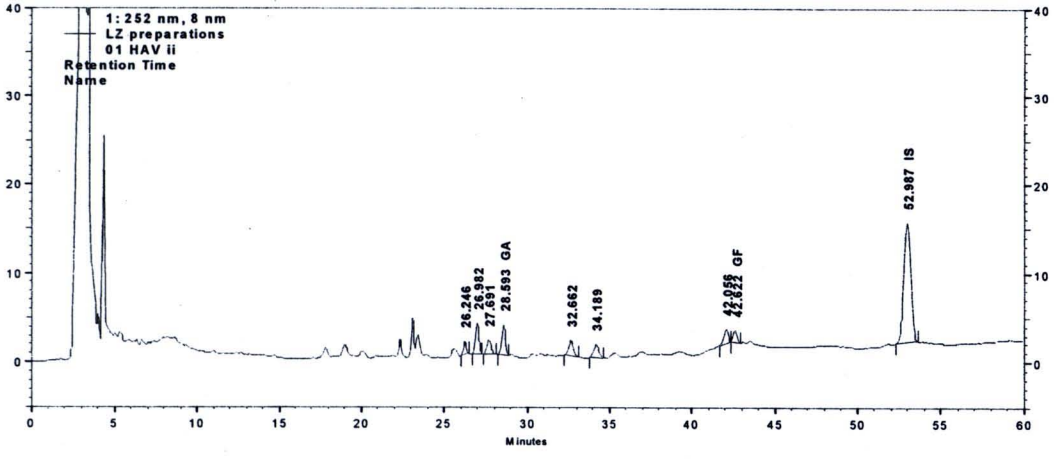


Figure 6 HPLC chromatograms of 19 Ling Zhi preparations used for determining ganoderic acids A (GA) and F (GF).

DHP-c



HAV



TYP

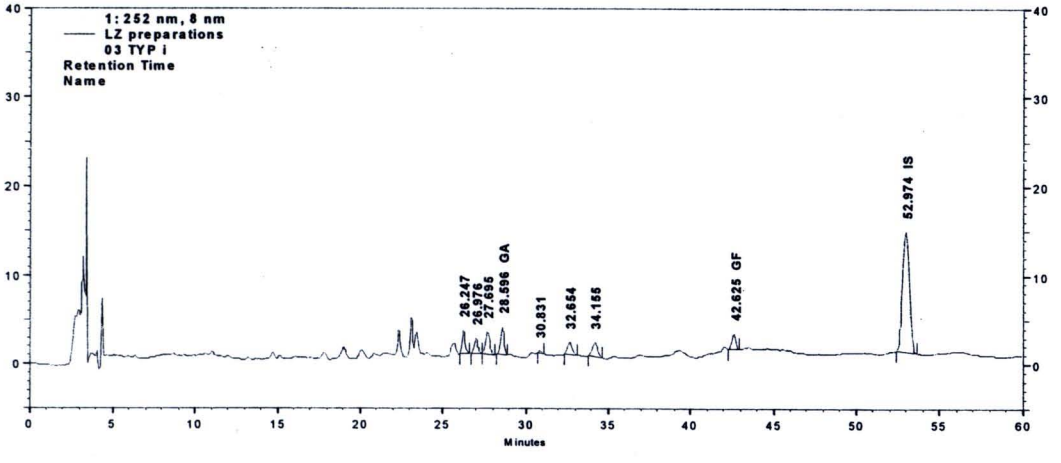
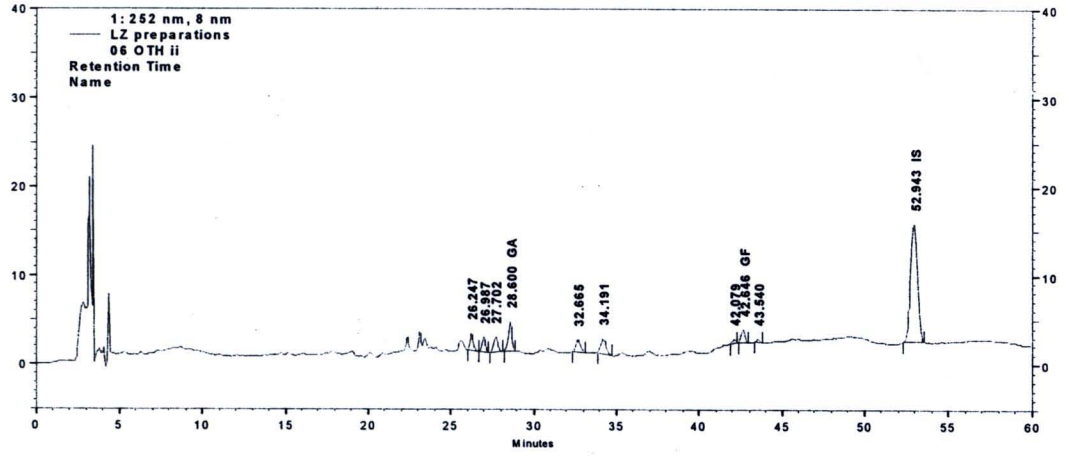
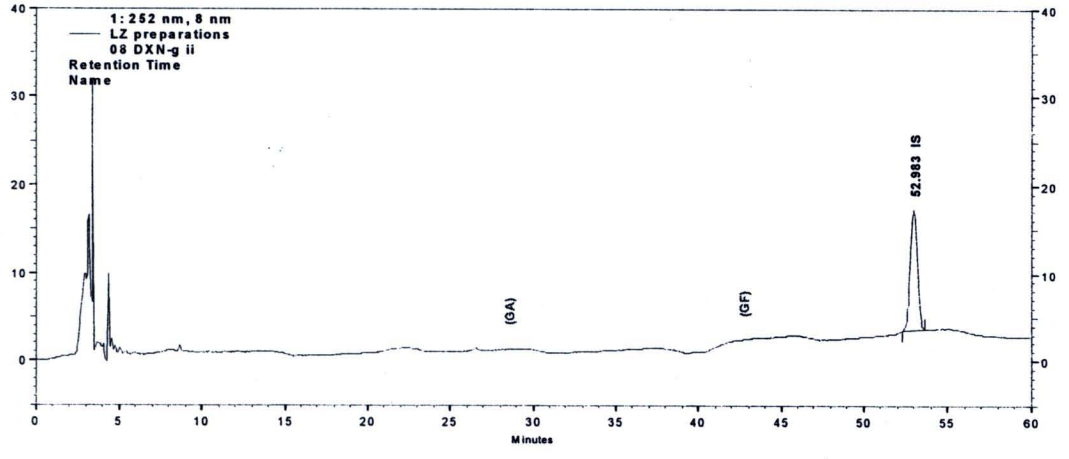


Figure 6 (Continued).

OTH



DXN-g



GEC

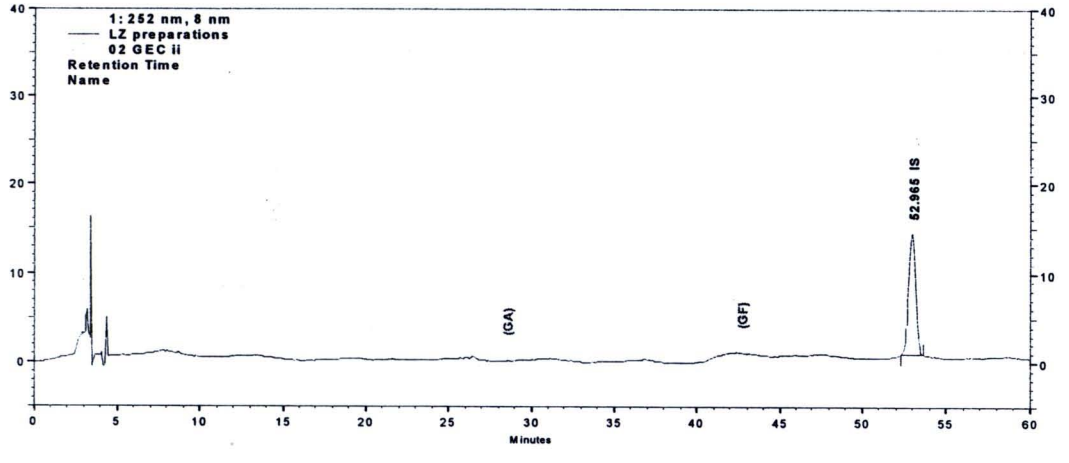
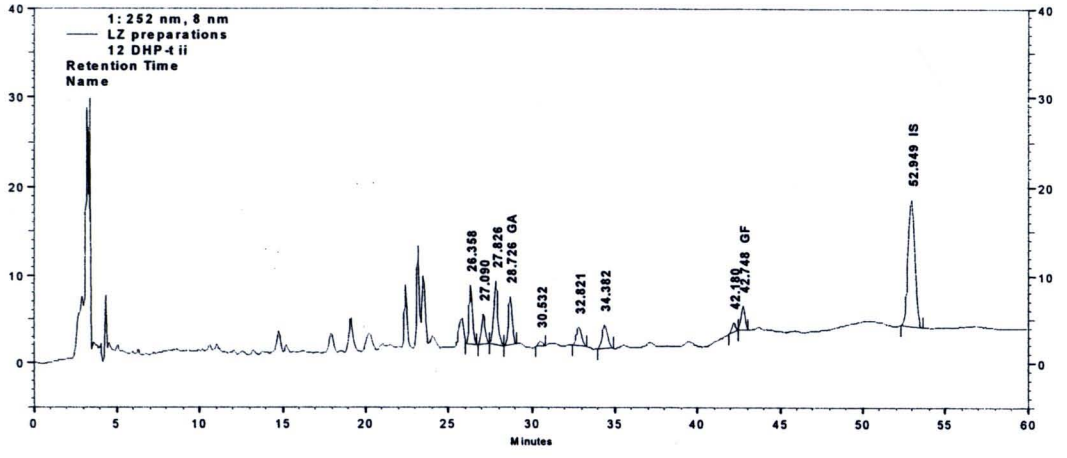
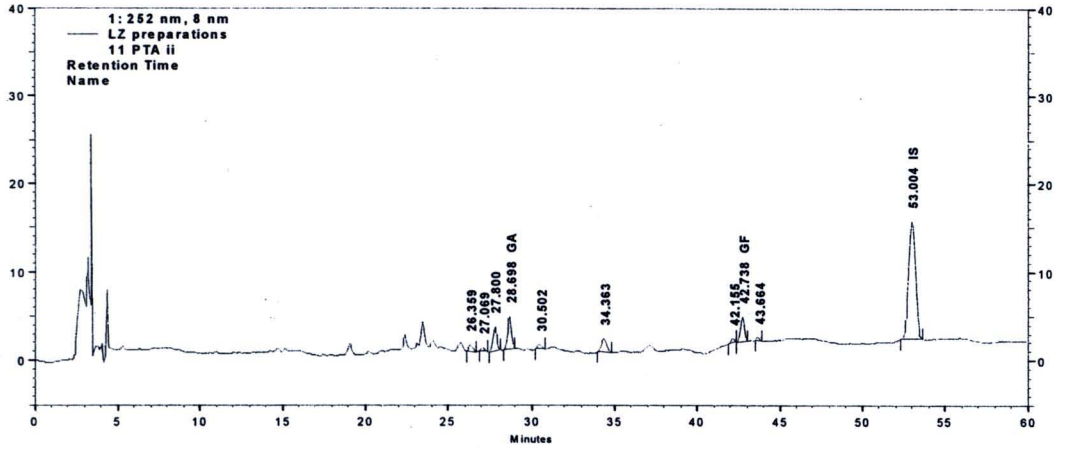


Figure 6 (Continued).

DHP-t



PTA



DTG-t

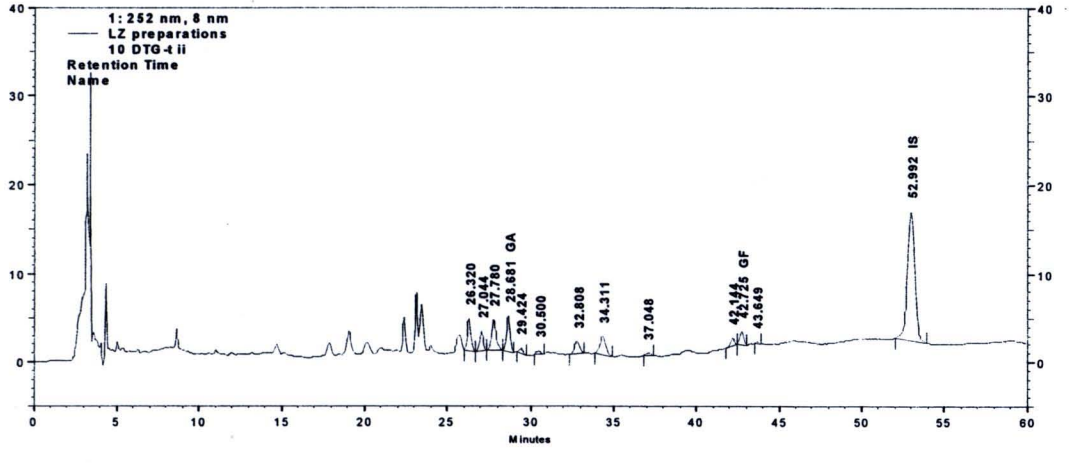
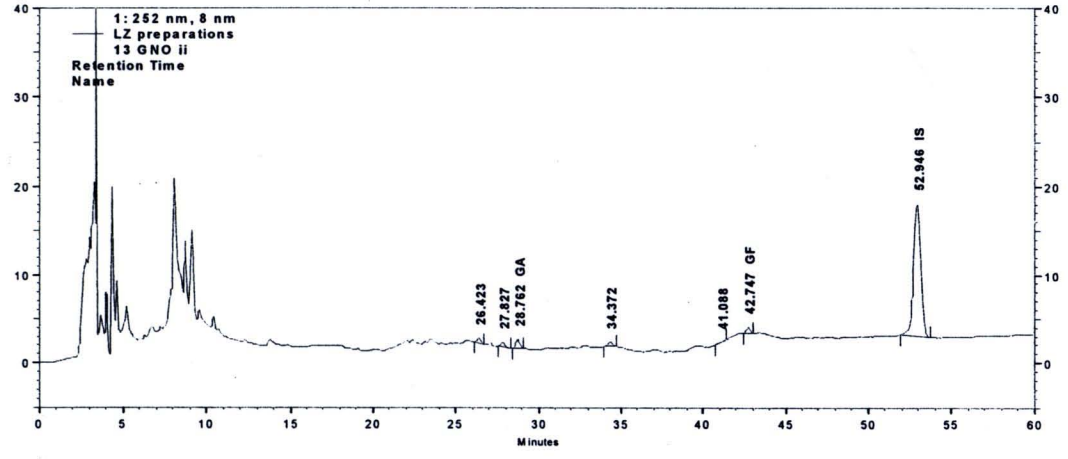
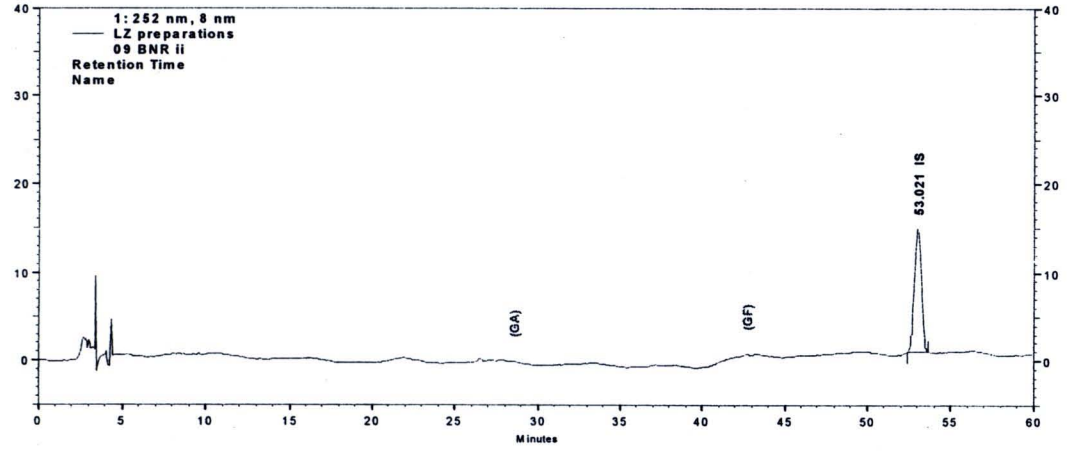


Figure 6 (Continued).

GNO



BNR



DTG-s

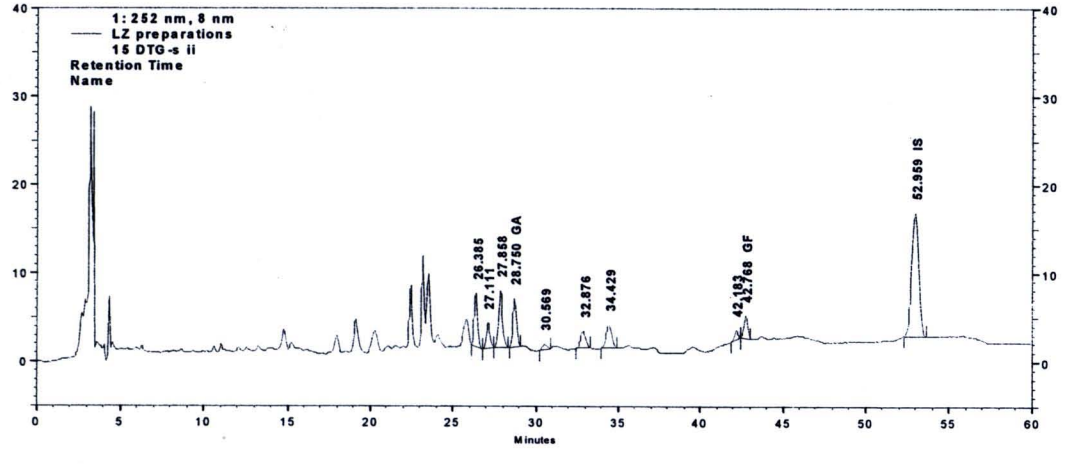
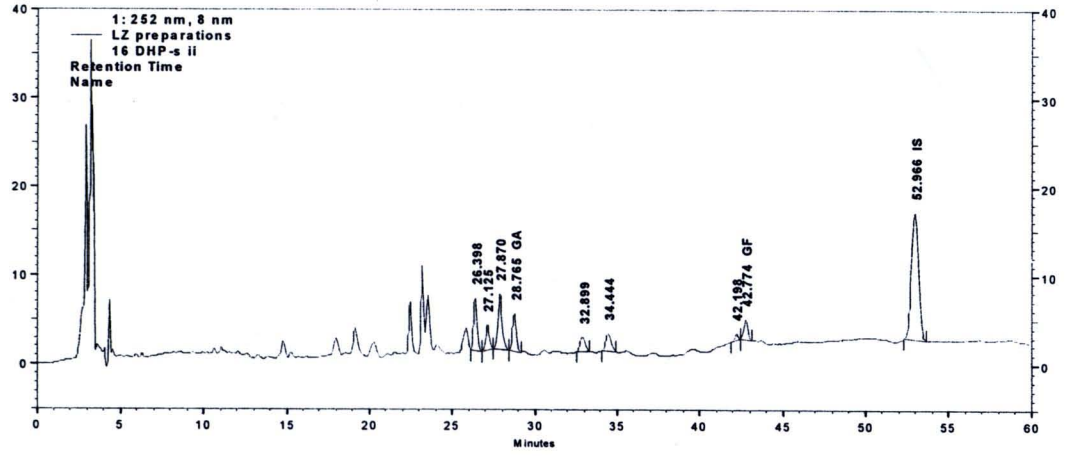
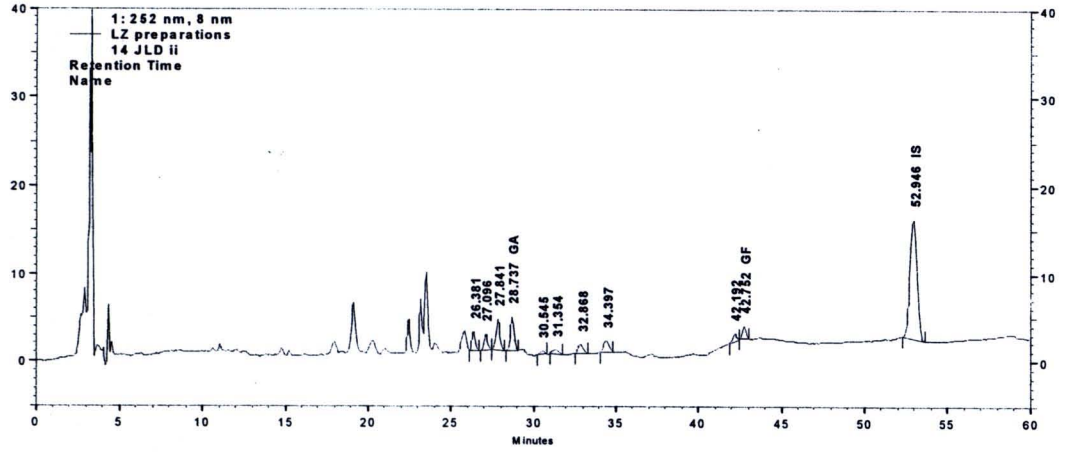


Figure 6 (Continued).

DHP-s



JLD



MG2FB

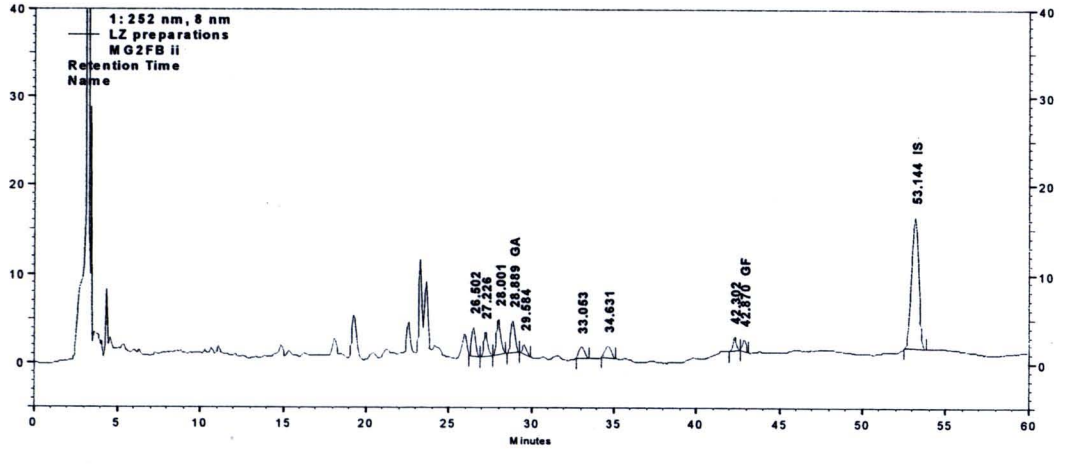
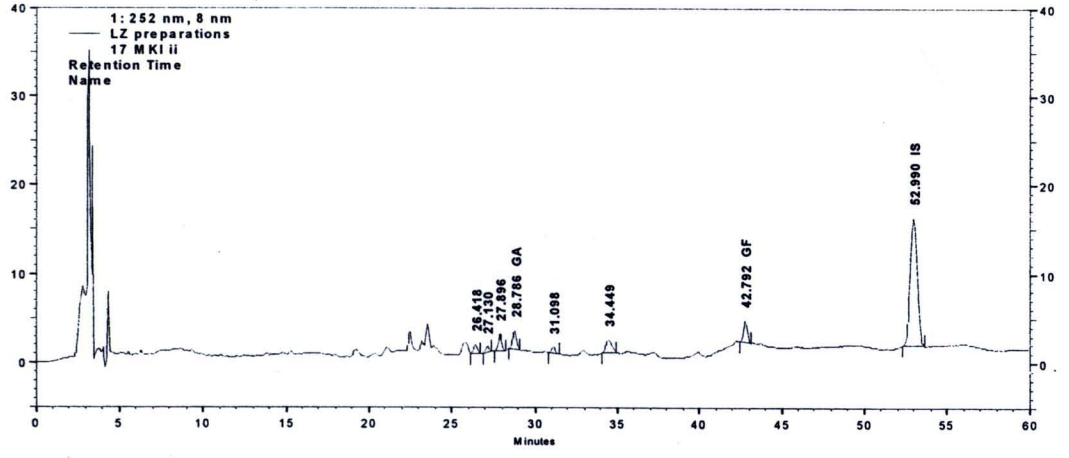


Figure 6 (Continued).

MKI



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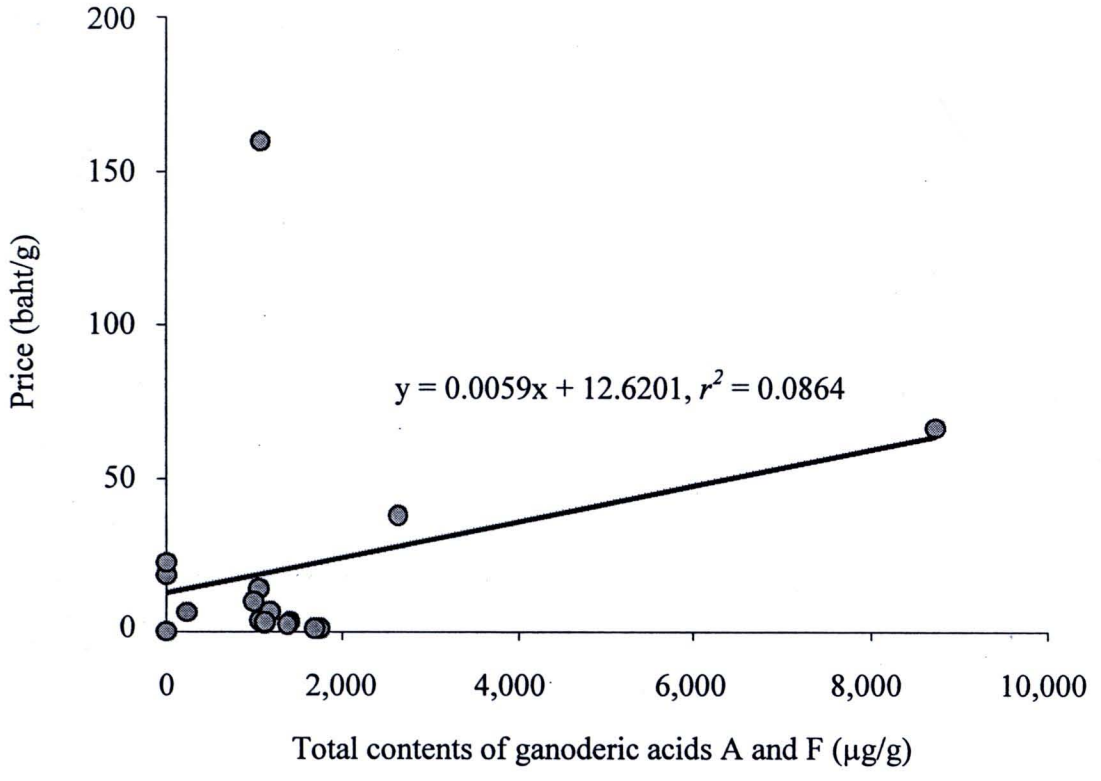

Figure 7 Correlation between the total contents of ganoderic acids A and F in 17 commercially available Ling Zhi preparations and their prices.

3.2 Pharmacokinetic study of ganoderic acids A and F after an oral administration of Ling Zhi preparation

3.2.1 Subjects

Twelve healthy Thai male subjects were included in this study and all subjects completed the study protocol. On the basis of medical history, physical examination and laboratory investigation, none of the subjects showed any evidence of neurological, pulmonary, kidney, liver or cardiovascular diseases. The demographic characteristics and clinical laboratory data of all subjects are shown in Table 5.

Table 5 The demographic characteristics and clinical laboratory data of 12 subjects participated in this study

Parameters	Mean ± SD	Range	Normal values
Age (y)	26.83 ± 4.86	20-33	
Weight (kg)	58.38 ± 7.29	48.5-74.0	
Height (m)	1.69 ± 0.06	1.60-1.78	
BMI (kg/m^2)	20.51 ± 1.97	18.25-23.36	18-25
Laboratory data			
Hemoglobin (g/L)	145.50 ± 6.49	130-157	130-180
Hematocrit (L/L)	0.45 ± 0.02	0.43-0.48	0.40-0.54
WBC (x 10^9/L)	7.93 ± 1.83	5.6-11.0	4.4-11.0
Platelets on smear	Adequate	-	Adequate
BUN (mg/dL)	13.17 ± 1.95	10-17	8.4-21
Creatinine (mg/dL)	1.08 ± 0.11	0.9-1.3	0.8-1.3
SGOT (U/L)	21.58 ± 4.54	16-30	0-37
SGPT (U/L)	23.50 ± 9.13	13-39	0-41
ALP (U/L)	47.50 ± 11.02	30-64	53-128
Total bilirubin (mg/dL)	0.42 ± 0.23	0.2-0.9	0.1-1.2

BMI, body mass index; WBC, white blood cell; BUN, blood urea nitrogen; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; ALP, Alkaline phosphatase

3.2.2 Assay validation of LC-MS method

Specificity

LC-MS chromatogram of ganoderic acids and IS-free plasma is presented in Figure 8A, whereas chromatogram of plasma containing 20 ng/mL of ganoderic acids A and F as well as 2.5 ng/mL of IS is shown in Figure 8B. Under LC-MS condition used in this study, ganoderic acid A was firstly eluted, followed by IS and ganoderic acid F with the retention time of 5.30, 9.11 and 10.52 min, respectively. All peaks were clearly separated and no peak interference was observed.

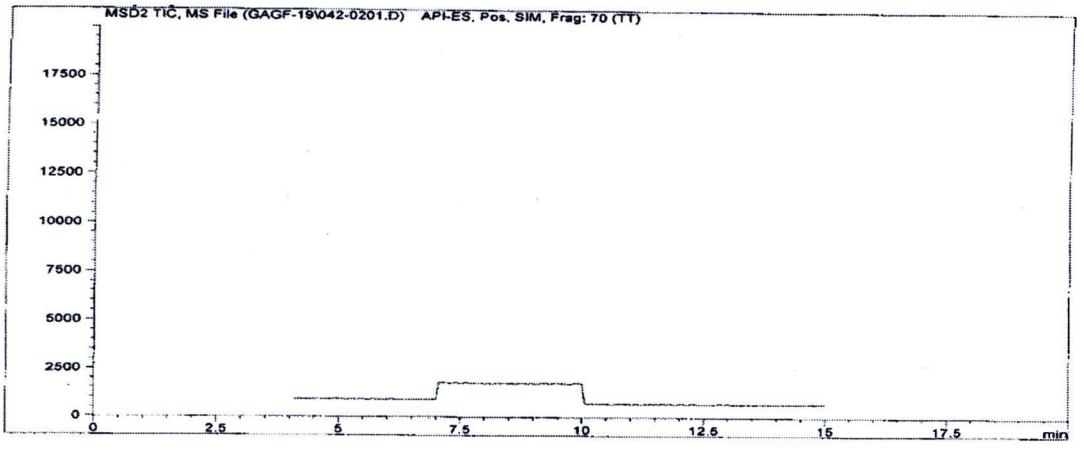

Figure 8A LC-MS chromatogram of ganoderic acids and IS-free plasma.

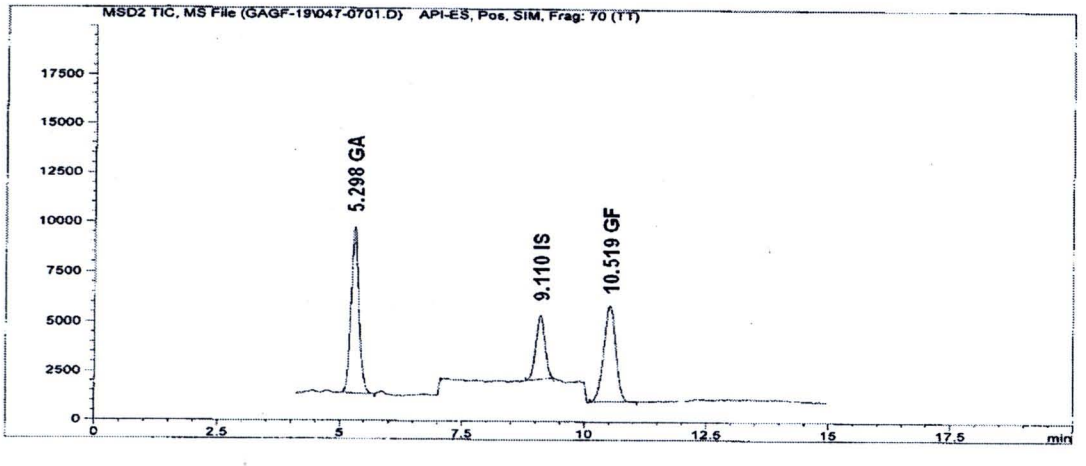

Figure 8B LC-MS chromatogram of plasma containing 18.00 ng/mL of ganoderic acids A (GA) and F (GF) as well as 2.50 ng/mL of IS.

LLOQ

LLOQ under the LC-MS condition used in this study was 0.50 ng/mL. The % CV at LLOQ concentration of ganoderic acids A and F were 5.92% and 15.90%, respectively, whereas the accuracy at this concentration were 113.50% and 114.95%, respectively (Table 6).

Table 6 The LLOQ of ganoderic acids A and F in plasma

No.	Spiked concentration (ng/mL)	Ganoderic acid A		Ganoderic acid F	
		Calculated concentration (ng/mL)	Accuracy (%)	Calculated concentration (ng/mL)	Accuracy (%)
1	0.50	0.61	122.24	0.45	89.85
2	0.50	0.52	104.94	0.53	106.53
3	0.50	0.54	108.91	0.64	128.29
4	0.50	0.58	115.88	0.68	136.31
5	0.50	0.58	115.54	0.57	113.79
	Mean	0.57	**113.50**	0.57	**114.95**
	SD	0.03		0.09	
	% CV	**5.92**		**15.90**	

Linearity of calibration curve

Pooled calibration curves from 5 replicated calibration data of the peak area ratios of ganoderic acid A/IS or ganoderic acid F/IS *versus* corresponding plasma ganoderic acid concentrations are shown in Figure 9A and 9B. All calibration curves exhibited good linearity within the test range (0.50-20.00 ng/mL) with $r^2 > 0.99$.

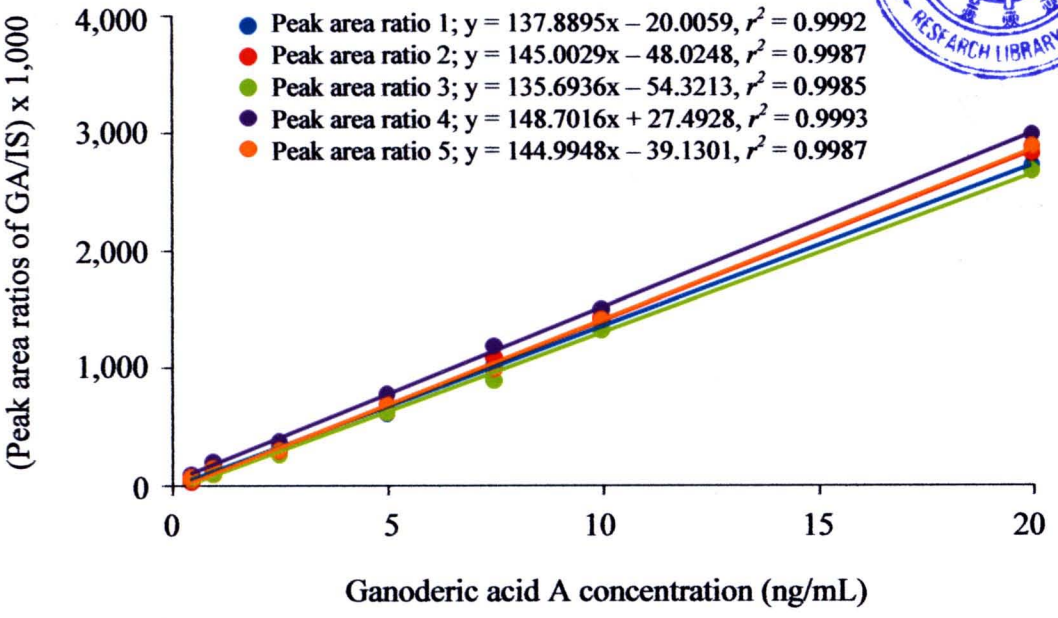

Figure 9A Pooled calibration curves from 5 replicated calibration data of peak area ratios of ganoderic acid A and IS *versus* plasma ganoderic acid A concentrations.

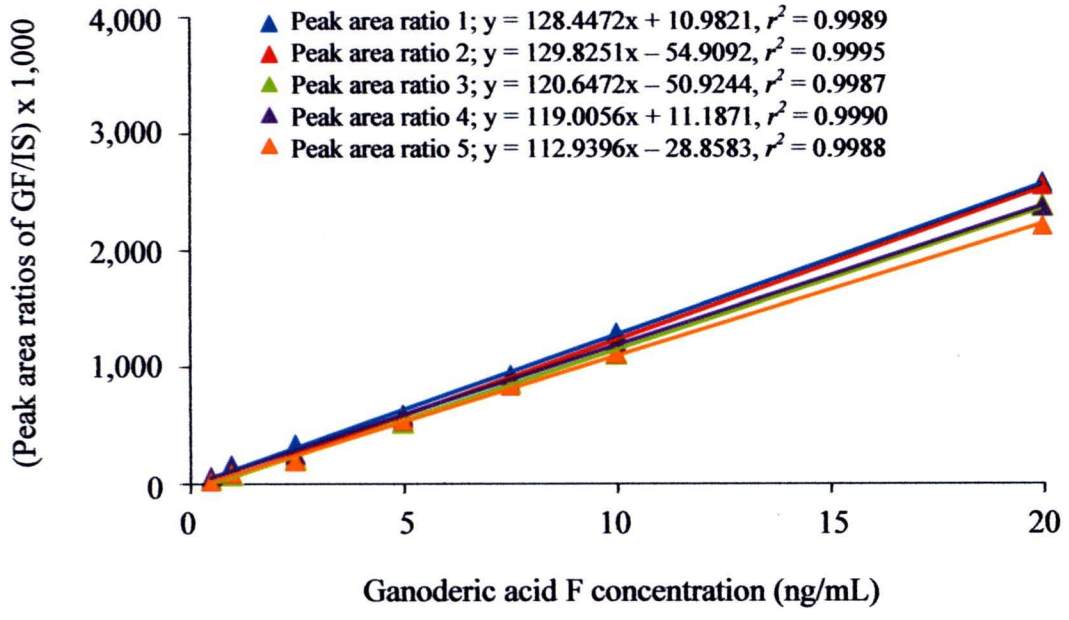

Figure 9B Pooled calibration curves from 5 replicated calibration data of peak area ratios of ganoderic acid F and IS *versus* plasma ganoderic acid F concentrations.

Precision and accuracy

The intra- and inter-day assay validation of ganoderic acids A and F in plasma are respectively presented in Table 7A and 7B. The % CV for low (1.50 ng/mL), medium (9.00 ng/mL), and high (18.00 ng/mL) concentrations of intra-day precision of ganoderic acids A and F ranged from 1.68-4.38% and 2.69-4.69%, respectively, whereas, those of inter-day of ganoderic acids A and F ranged from 3.28-3.84% and 1.55-4.03%, respectively. Likewise, the % accuracy of intra- and inter-day assay validation ranged from 100.74-106.74% for ganoderic acid A and 96.33-103.05% for ganoderic acid F. All these values were within the acceptable range of ± 15% precision and 85-115% of accuracy recommended by U.S. FDA guidance.

Table 7A Intra-day assay validation of ganoderic acids A and F in plasma

QC sample	Spiked concentration (ng/mL)	Peak area of IS	Ganoderic acid A				Ganoderic acid F			
			Peak area	GA/IS peak area ratio x 1000	Calculated concentration (ng/mL)	Accuracy (%)	Peak area	GF/IS peak area ratio x 1000	Calculated concentration (ng/mL)	Accuracy (%)
Low	1.50	56,573.70	10,332.70	182.64	1.59	106.24	8,469.34	149.70	1.56	103.83
	1.50	57,940.30	9,700.67	167.43	1.47	98.15	9,616.09	165.97	1.61	107.33
	1.50	55,134.60	9,902.84	179.61	1.57	104.63	7,742.11	140.42	1.47	97.98
	1.50	56,102.80	11,227.20	200.12	1.65	110.00	7,890.77	140.65	1.47	98.12
	1.50	51,890.80	10,235.50	197.25	1.63	108.67	8,668.30	167.05	1.62	108.00
	Mean	55,528.44	10,279.78	185.41	1.58	**105.54**	8,477.32	152.76	1.55	**103.05**
	SD	2,270.85	578.33	13.43	0.07		744.63	13.10	0.07	
	% CV				**4.38**				**4.69**	
Medium	9.00	48,129.20	57,330.90	1,191.19	9.64	107.11	49,758.30	1,033.85	9.20	102.19
	9.00	49,128.70	58,384.80	1,188.41	9.62	106.86	52,351.00	1,065.59	9.46	105.11
	9.00	52,900.80	57,269.80	1,082.59	8.77	97.48	50,847.80	961.19	8.60	95.52
	9.00	51,284.10	60,081.20	1,171.54	9.48	105.36	52,076.50	1,015.45	9.05	100.50
	9.00	51,523.30	56,791.10	1,102.24	8.93	99.22	52,037.60	1,009.98	9.00	100.00
	Mean	50,593.22	57,971.56	1,147.19	9.29	**103.21**	51,414.24	1,017.21	9.06	**100.66**
	SD	1,928.96	1,315.08	51.04	0.41		1,091.75	38.11	0.32	
	% CV				**4.38**				**3.48**	
High	18.00	52,589.80	123,827.00	2,354.58	18.92	105.11	105,687.00	2,009.65	17.26	95.90
	18.00	55,196.20	134,832.00	2,442.78	19.42	107.89	119,058.00	2,157.00	18.48	102.67
	18.00	51,138.90	123,204.00	2,409.20	19.36	107.54	107,674.00	2,105.52	18.05	100.30
	18.00	52,744.80	131,010.00	2,483.85	19.55	108.61	113,323.00	2,148.52	18.41	102.28
	18.00	51,940.30	121,657.00	2,342.25	18.82	104.57	110,109.00	2,119.91	18.17	100.97
	Mean	52,722.00	126,906.00	2,406.53	19.21	**106.74**	111,170.20	2,108.12	18.08	**100.42**
	SD	1,521.26	5,709.67	59.43	0.32		5,250.40	58.87	0.49	
	% CV				**1.68**				**2.69**	
	Average				**3.48***	**105.16#**			**3.62***	**101.38#**

* Average % CV = (the sum of % CV at low, medium and high concentrations)/3

Average % accuracy = (the sum of mean values of % accuracy at low, medium and high concentrations)/3

Table 7B Inter-day assay validation of ganoderic acids A and F in plasma

Spiked concentration (ng/mL)	Ganoderic acid A							Ganoderic acid F						
	Calculated concentration (ng/mL)					Precision (% CV)	Accuracy (%)	Calculated concentration (ng/mL)					Precision (% CV)	Accuracy (%)
	Day 1	Day 2	Day 3	Mean	SD			Day 1	Day 2	Day 3	Mean	SD		
1.50 (n = 5)	1.61 ± 0.13	1.58 ± 0.07	1.51 ± 0.11	1.57	0.05	3.28	104.44	1.54 ± 0.12	1.55 ± 0.07	1.44 ± 0.07	1.51	0.06	4.03	100.67
9.00 (n = 5)	9.24 ± 0.31	9.29 ± 0.41	8.67 ± 0.54	9.07	0.34	3.80	100.74	8.53 ± 0.37	9.06 ± 0.32	8.42 ± 0.58	8.67	0.34	3.95	96.33
18.00 (n = 5)	18.99 ± 0.67	19.21 ± 0.32	17.87 ± 0.84	18.69	0.72	3.84	103.83	17.98 ± 0.81	18.08 ± 0.49	18.51 ± 0.75	18.19	0.28	1.55	101.06
	Average					**3.64***	**103.01#**	Average					**3.17***	**99.35#**

* Average % CV = (the sum of % CV at the concentrations of 1.50, 9.00 and 18.00 ng/mL)/3

Average % accuracy = (the sum of % accuracy at the concentrations of 1.50, 9.00 and 18.00 ng/mL)/3

Recovery

The recovery of plasma extraction method of ganoderic acids A and F as well as IS is shown in Table 8. At low (1.50 ng/mL), medium (9.00 ng/mL), and high (18.00 ng/mL) concentrations, the % recovery of ganoderic acids A and F ranged from 72.27-75.17%, and 85.66-92.45%, respectively, whereas, those of IS ranged from 70.71-74.37%. The overall average values of % recovery of ganoderic acids A and F including IS were 73.51%, 89.52% and 72.66%, respectively.

Table 8 Recovery of ganoderic acids A and F and IS in plasma

QC sample	Spiked concentration (ng/mL)	Ganoderic acid A (Peak area)		Ganoderic acid F (Peak area)		IS (Peak area)	
		In mobile phase	After plasma extraction	In mobile phase	After plasma extraction	In mobile phase	After plasma extraction
Low	1.50	18,718.80	13,807.80	11,295.70	9,079.17	76,939.20	58,058.40
	1.50	19,091.10	12,687.30	10,268.10	7,751.11	80,520.70	60,172.10
	1.50	17,832.90	13,560.70	9,322.89	8,994.31	85,088.40	57,689.80
	1.50	16,950.70	12,443.50	7,463.60	8,182.69	83,964.90	60,343.90
	1.50	16,953.40	12,218.30	8,973.14	8,807.22	86,657.50	64,991.50
	Mean	17,909.38	12,943.52	9,464.69	8,562.90	82,634.14	60,251.14
	SD	986.21	701.70	1,437.37	573.56	3,901.30	2,909.23
	% Recovery*		**72.27**		**90.47**		**72.91**
Medium	9.00	82,790.40	64,925.70	57,850.10	52,839.80	89,281.90	62,688.20
	9.00	83,048.00	64,711.30	57,292.60	55,808.30	87,589.80	67,626.70
	9.00	83,988.00	63,820.10	60,421.20	54,671.70	88,320.00	64,723.10
	9.00	91,249.10	66,095.40	59,924.20	53,345.30	88,160.40	68,942.60
	9.00	88,623.00	63,462.40	58,851.90	55,439.80	90,588.60	66,162.70
	Mean	85,939.70	64,602.98	58,868.00	54,420.98	88,788.14	66,028.66
	SD	3,790.74	1,031.63	1,327.24	1,292.55	1,176.30	2,445.71
	% Recovery*		**75.17**		**92.45**		**74.37**
High	18.00	189,693.00	138,578.00	146,799.00	119,534.00	91,710.90	64,359.50
	18.00	190,233.00	131,884.00	144,166.00	118,857.00	93,428.40	66,725.50
	18.00	177,723.00	133,343.00	139,755.00	120,800.00	98,847.80	64,184.60
	18.00	181,160.00	134,940.00	138,205.00	122,803.00	89,759.10	64,074.60
	18.00	180,057.00	132,694.00	139,256.00	124,611.00	90,135.30	68,678.60
	Mean	183,773.20	134,287.80	141,636.20	121,321.00	92,776.30	65,604.56
	SD	5,788.28	2,647.75	3,675.32	2,374.69	3,691.27	2,037.98
	% Recovery*		**73.07**		**85.66**		**70.71**
Average recovery (%)#			**73.51**		**89.52**		**72.66**

* % Recovery = (mean value of peak area after plasma extraction/mean value of peak area in mobile phase) x 100

Average recovery (%) = (the sum of % recovery at low, medium and high concentrations)/3

Freeze/thaw stability

The stability of ganoderic acids A and F in plasma at low (1.50 ng/mL) and high (18.00 ng/mL) concentrations kept frozen at -20 °C before and after freeze and thaw test is presented in Table 9. The % remaining of ganoderic acid A at low and high concentrations after three unassisted freeze/thaw cycles was 93.53% and 91.34%, respectively, whereas that of ganoderic acid F was 98.61% and 92.39%, respectively. The average freeze/thaw stability of ganoderic acids A and F was 92.43% and 95.50%, respectively.

Table 9 Freeze/thaw stability of ganoderic acids A and F in plasma

QC sample	Spiked concentration (ng/mL)	Ganoderic acid A concentration (ng/mL)		Ganoderic acid F concentration (ng/mL)	
		Before freeze/thaw	After freeze/thaw	Before freeze/thaw	After freeze/thaw
Low	1.50	1.38	1.31	1.45	1.42
	1.50	1.47	1.34	1.40	1.38
	1.50	1.48	1.40	1.48	1.47
	Mean	1.44	1.35	1.44	1.42
	SD	0.06	0.05	0.04	0.05
	% Remaining*		**93.53**		**98.61**
High	18.00	17.06	16.10	17.87	17.21
	18.00	18.11	16.18	18.70	16.72
	18.00	18.38	16.63	19.05	17.46
	Mean	17.85	16.30	18.54	17.13
	SD	0.70	0.29	0.61	0.38
	% Remaining*		**91.34**		**92.39**
Average freeze/thaw stability (%)[#]			**92.43**		**95.50**

* % Remaining = (mean value of concentration after 3 freeze/thaw cycles)/(mean value of concentration before freeze/thaw) x 100

[#] Average freeze/thaw stability (%) = (the sum of % remaining at low and high concentrations)/2

Short-term stability

Short-term stability of ganoderic acids A and F in plasma at low (1.50 ng/mL) and high (18.00 ng/mL) concentrations kept at room temperature (t_{room}) for 8 h against those of freshly prepared samples is shown in Table 10. The % remaining of ganoderic acid A at low and high concentrations kept at t_{room} for 8 h was 90.06% and 97.52%, respectively, whereas that of ganoderic acid F was 91.59% and 96.57%, respectively. The average short-term stability of ganoderic acids A and F was 93.79% and 94.08%, respectively.

Table 10 Short-term stability of ganoderic acids A and F in plasma

QC sample	Spiked concentration (ng/mL)	Ganoderic acid A concentration (ng/mL)		Ganoderic acid F concentration (ng/mL)	
		Fresh preparation	Kept at t_{room} for 8 h	Fresh preparation	Kept at t_{room} for 8 h
Low	1.50	1.47	1.33	1.61	1.41
	1.50	1.57	1.42	1.47	1.39
	1.50	1.59	1.42	1.56	1.45
	Mean	1.54	1.39	1.55	1.42
	SD	0.06	0.05	0.07	0.03
	% Remaining*		**90.06**		**91.59**
High	18.00	18.82	18.04	18.17	16.77
	18.00	18.92	18.34	17.26	16.90
	18.00	19.36	19.30	18.05	17.98
	Mean	19.03	18.56	17.83	17.22
	SD	0.29	0.66	0.49	0.67
	% Remaining*		**97.52**		**96.57**
Average short-term stability (%)#			**93.79**		**94.08**

* % Remaining = (mean value of concentration kept at t_{room} for 8 h)/(mean value of concentration after fresh preparation) x 100

\# Average short-term stability (%) = (the sum of % remaining at low and high concentrations)/2

Long-term stability

Table 11 shows the long-term stability of ganoderic acids A and F in plasma at low (1.50 ng/mL) and high (18.00 ng/mL) concentrations kept at -20 °C for 1.5 months with those from the first day of this testing. The % remaining of ganoderic acid A at low and high concentrations maintained under the same conditions as the study samples for long period was 96.82% and 101.07%, respectively, whereas that of ganoderic acid F was 98.30% and 98.28%, respectively. The average long-term stability of ganoderic acids A and F was 98.95% and 98.29%, respectively.

Table 11 Long-term stability of ganoderic acids A and F in plasma

QC sample	Spiked concentration (ng/mL)	Ganoderic acid A concentration (ng/mL)		Ganoderic acid F concentration (ng/mL)	
		Day 1 of testing	Month 1.5 of testing	Day 1 of testing	Month 1.5 of testing
Low	1.50	1.56	1.61	1.57	1.58
	1.50	1.63	1.43	1.59	1.54
	1.50	1.53	1.53	1.54	1.50
	Mean	1.57	1.52	1.57	1.54
	SD	0.05	0.09	0.03	0.04
	% Remaining*		**96.82**		**98.30**
High	18.00	18.01	18.10	19.09	18.28
	18.00	18.08	18.44	19.45	18.37
	18.00	19.04	19.18	18.60	19.51
	Mean	18.38	18.57	19.05	18.72
	SD	0.58	0.55	0.43	0.69
	% Remaining*		**101.07**		**98.28**
Average long-term stability (%)#			**98.95**		**98.29**

* % Remaining = (mean value of concentration kept at -20 °C for 1.5 months)/(mean value of concentration at the first day of testing) x 100

Average long-term stability (%) = (the sum of % remaining at low and high concentrations)/2

Post-preparative stability

Post-preparative stability of ganoderic acids A and F in plasma at low (1.50 ng/mL) and high (18.00 ng/mL) concentrations standing in the autosampler for 18 h after preparation against those of freshly prepared samples is presented in Table 12. The % remaining of ganoderic acid A at low and high concentrations after waiting in the autosampler was 91.45% and 92.90%, respectively, whereas that of ganoderic acid F was 105.75% and 97.26%, respectively. The average post-preparative stability of ganoderic acids A and F was 92.17% and 101.50%, respectively.

Table 12 Post-preparative stability of ganoderic acids A and F in plasma

QC sample	Spiked concentration (ng/mL)	Ganoderic acid A concentration (ng/mL)		Ganoderic acid F concentration (ng/mL)	
		Fresh preparation	18 h after preparation	Fresh preparation	18 h after preparation
Low	1.50	1.49	1.36	1.53	1.57
	1.50	1.60	1.30	1.40	1.62
	1.50	1.43	1.47	1.42	1.41
	Mean	1.50	1.38	1.45	1.53
	SD	0.08	0.09	0.07	0.11
	% Remaining*		**91.45**		**105.75**
High	18.00	17.94	15.34	18.10	15.83
	18.00	17.39	17.84	18.69	20.59
	18.00	17.57	15.96	18.79	17.63
	Mean	17.63	16.38	18.53	18.02
	SD	0.28	1.30	0.38	2.40
	% Remaining*		**92.90**		**97.26**
Average post-preparative stability (%)#			**92.17**		**101.50**

* % Remaining = (mean value of concentration standing in autosampler for 18 h after preparation)/(mean value of concentration after fresh preparation) x 100

Average post-preparative stability (%) = (the sum of % remaining at low and high concentrations)/2

3.2.3 Pharmacokinetic parameters of ganoderic acids A and F

The individual as well as mean plasma concentration-time profiles of ganoderic acid A at various sampling times from 12 subjects after a single oral administration of 3,000 mg of MG2FB-WE under fasted or fed condition are respectively presented in Table 13A and 13B. Those of ganoderic acid F under fasted condition are presented in Table 13C. However, plasma ganoderic acid F concentrations that are higher than the LLOQ under fed condition were detected only in 2 out of 12 subjects (subjects No.3 and 11). In one subject, the concentrations of 0.59 and 0.50 ng/mL were found at 2.50 h and 3.00 h post-dose, respectively, whereas the concentration of 0.56 ng/mL was found at 3.50 h post-dose in another subject. The plasma concentration-time curves of ganoderic acids A and F in both conditions from each subject are depicted in Figure 10.

Composites of individual plasma ganoderic acids A and F concentration-time curves from 12 subjects after a single oral dose of MG2FB-WE under fasted or fed condition are sequentially presented in Figure 11A-D. Their mean plasma concentration-time curves in both conditions are also shown in Figure 12A and 12B, respectively.

Table 13A Plasma concentrations of ganoderic acid A (ng/mL) after a single oral dose of MG2FB-WE under fasted condition in 12 subjects

Subject	Plasma concentrations of ganoderic acid A (ng/mL) at various sampling times (h)															
No.	0.00	0.08	0.17	0.25	0.50	0.75	1.00	1.50	2.00	2.50	3.00	3.50	4.00	5.00	6.00	8.00
1	BLQ	1.40	3.92	8.74	5.60	2.63	1.69	0.86	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
2	BLQ	BLQ	1.00	4.44	5.36	2.80	1.43	0.53	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
3	BLQ	1.45	3.57	5.00	15.15	13.13	10.54	3.27	2.18	1.33	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
4	BLQ	BLQ	0.76	1.53	4.82	4.97	5.53	2.17	1.31	0.98	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	0.58	2.35	3.81	12.29	5.35	2.85	1.29	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
6	BLQ	0.56	2.42	9.78	16.31	10.03	6.67	2.12	1.10	0.86	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
7	BLO	0.67	1.17	5.63	10.72	7.89	5.86	2.05	1.24	0.58	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
8	BLQ	0.78	2.99	5.70	7.91	3.34	2.24	1.19	0.82	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
9	BLQ	BLQ	1.72	7.42	12.48	11.55	7.38	3.17	2.99	1.14	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
10	BLQ	2.35	3.17	4.63	6.79	7.17	5.93	2.95	2.13	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
11	BLQ	BLQ	1.72	7.17	13.88	10.74	7.22	2.06	1.41	0.74	0.68	BLQ	BLQ	BLQ	BLQ	BLQ
12	BLQ	2.07	3.18	6.17	16.36	11.05	7.28	4.33	2.12	1.28	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Mean	BLQ	1.23*	2.33	5.84	10.64	7.56	5.38	2.17	1.70*	0.99*	ID	BLQ	BLQ	BLQ	BLQ	BLQ
SD	BLQ	0.70	1.05	2.24	4.38	3.72	2.79	1.11	0.69	0.28	ID	BLQ	BLQ	BLQ	BLQ	BLQ
% CV	BLQ	56.73	45.23	38.41	41.20	49.20	51.72	51.47	40.70	28.04	ID	BLQ	BLQ	BLQ	BLQ	BLQ

BLQ = Below the lower limit of quantification

ID = Insufficient data (n = 1) for reliable calculation

* Plasma samples with BLQ were not taken into account

Table 13B Plasma concentrations of ganoderic acid A (ng/mL) after a single oral dose of MG2FB-WE under fed condition in 12 subjects

Subject	Plasma concentrations of ganoderic acid A (ng/mL) at various sampling times (h)															
No.	0.00	0.08	0.17	0.25	0.50	0.75	1.00	1.50	2.00	2.50	3.00	3.50	4.00	5.00	6.00	8.00
1	BLQ	BLQ	BLQ	0.54	1.09	1.15	1.17	1.18	1.38	1.42	2.58	1.96	1.86	0.84	BLQ	BLQ
2	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	0.73	1.99	3.25	1.73	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
3	BLQ	BLQ	0.76	1.50	2.91	3.34	3.48	4.03	4.29	3.93	2.61	2.31	1.89	0.74	BLQ	BLQ
4	BLQ	BLQ	1.76	3.26	5.08	4.37	4.25	3.34	1.59	1.32	0.63	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	0.55	1.57	1.84	2.20	1.72	1.15	BLQ	BLQ	BLQ
6	BLQ	BLQ	BLQ	BLQ	0.87	1.35	2.44	2.54	2.46	2.17	1.94	1.87	1.32	BLQ	BLQ	BLQ
7	BLQ	BLQ	1.40	1.97	3.81	4.05	3.78	3.28	1.51	1.21	1.15	1.11	0.84	BLO	BLO	BLQ
8	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	2.14	3.22	1.54	1.18	1.05	BLQ	BLQ	BLQ
9	BLQ	BLQ	0.90	1.62	3.76	4.76	5.58	4.62	3.56	2.85	1.71	1.42	1.53	BLQ	BLQ	BLQ
10	BLQ	BLQ	0.56	1.34	2.30	2.49	2.38	2.37	2.11	1.37	0.94	BLQ	BLQ	BLQ	BLQ	BLQ
11	BLQ	BLQ	BLQ	BLQ	1.38	2.72	3.33	3.10	2.90	2.35	2.03	1.97	1.79	BLQ	BLQ	BLQ
12	BLQ	BLQ	1.04	1.78	3.23	4.74	5.58	7.09	7.45	4.88	4.61	4.09	2.75	1.68	0.64	BLQ
Mean	BLQ	BLQ	1.07*	1.72*	2.71*	3.22*	3.27*	3.10*	2.85	2.36	1.99*	1.96*	1.58*	1.09*	ID	BLQ
SD	BLQ	BLQ	0.44	0.82	1.42	1.38	1.64	1.77	1.71	1.16	1.07	0.89	0.58	0.52	ID	BLQ
% CV	BLQ	BLQ	41.34	47.72	52.41	42.90	50.24	57.21	60.09	49.12	53.84	45.44	36.77	47.73	ID	BLQ

BLQ = Below the lower limit of quantification

ID = Insufficient data (n = 1) for reliable calculation

* Plasma samples with BLQ were not taken into account

Table 13C Plasma concentrations of ganoderic acid F (ng/mL) after a single oral dose of MG2FB-WE under fasted condition in 12 subjects

Subject No.	Plasma concentrations of ganoderic acid F (ng/mL) at various sampling times (h)															
	0.00	0.08	0.17	0.25	0.50	0.75	1.00	1.50	2.00	2.50	3.00	3.50	4.00	5.00	6.00	8.00
1	BLQ	BLQ	1.33	3.24	1.49	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
2	BLQ	BLQ	BLQ	0.91	1.05	BLQ	0.57	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
3	BLQ	BLQ	BLQ	0.67	2.55	2.61	2.16	0.65	0.93	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
4	BLQ	BLQ	BLQ	BLQ	1.65	0.60	1.39	0.70	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	2.49	1.07	0.76	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
6	BLQ	BLQ	0.63	2.88	4.16	2.65	1.58	0.58	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
7	BLQ	BLQ	BLQ	0.72	1.02	1.17	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
8	BLQ	BLQ	1.42	1.84	2.56	1.08	0.58	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
9	BLQ	BLQ	BLQ	1.67	2.73	1.76	1.37	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
10	BLQ	BLQ	0.86	1.43	2.62	2.33	2.19	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
11	BLQ	BLQ	BLQ	2.34	3.33	1.72	0.62	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
12	BLQ	BLQ	BLQ	BLQ	3.25	0.84	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Mean	BLQ	BLQ	1.06*	1.74*	2.41	1.58*	1.25*	0.64*	ID	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
SD	BLQ	BLQ	0.38	0.93	0.95	0.75	0.65	0.06	ID	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
% CV	BLQ	BLQ	35.76	53.23	39.62	47.16	52.36	9.71	ID	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

BLQ = Below the lower limit of quantification

ID = Insufficient data (n = 1) for reliable calculation

* Plasma samples with BLQ were not taken into account

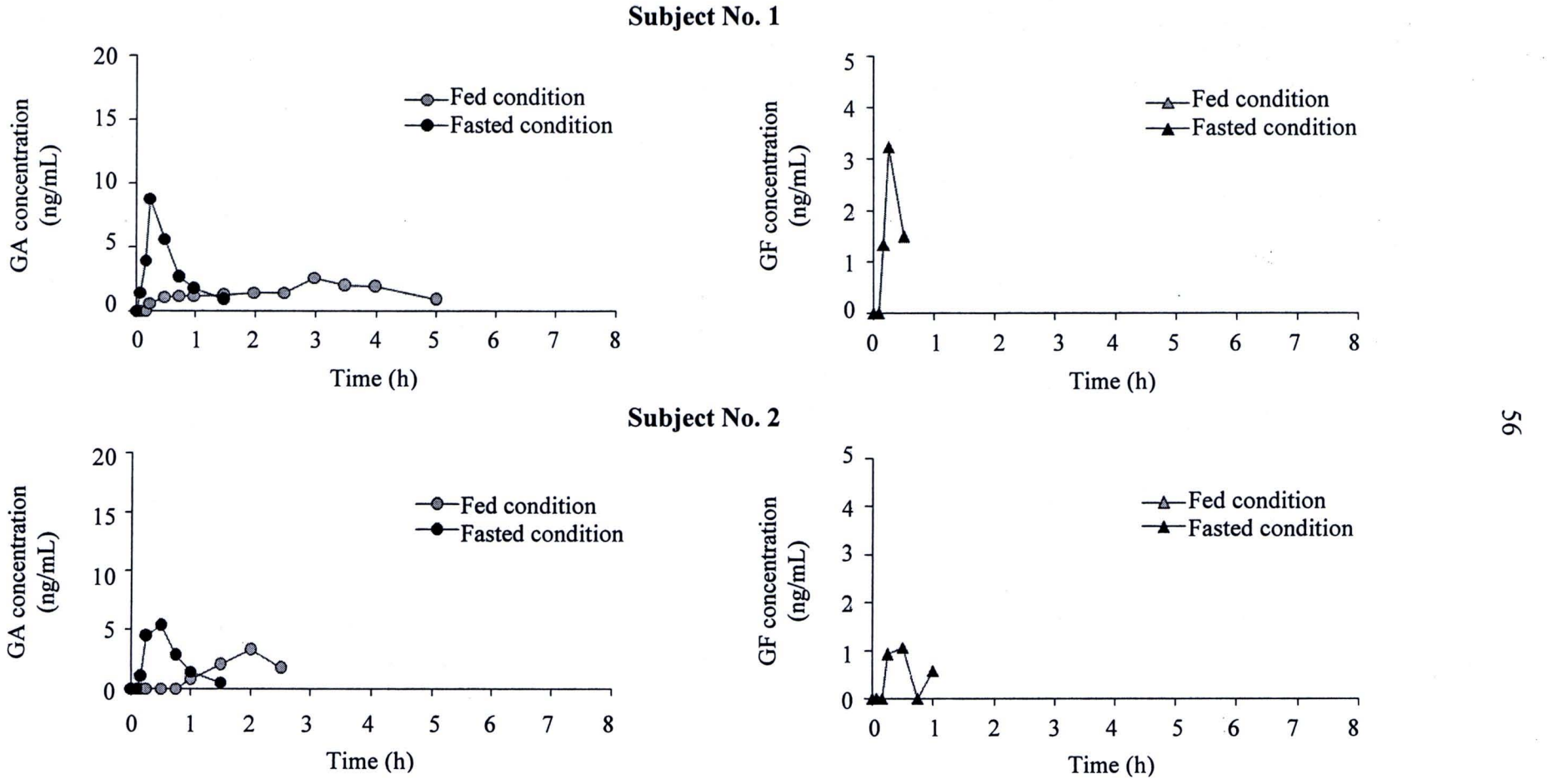

Figure 10 Plasma concentration-time curves of ganoderic acids A (GA) and F (GF) after a single oral dose of MG2FB-WE under fasted or fed condition.

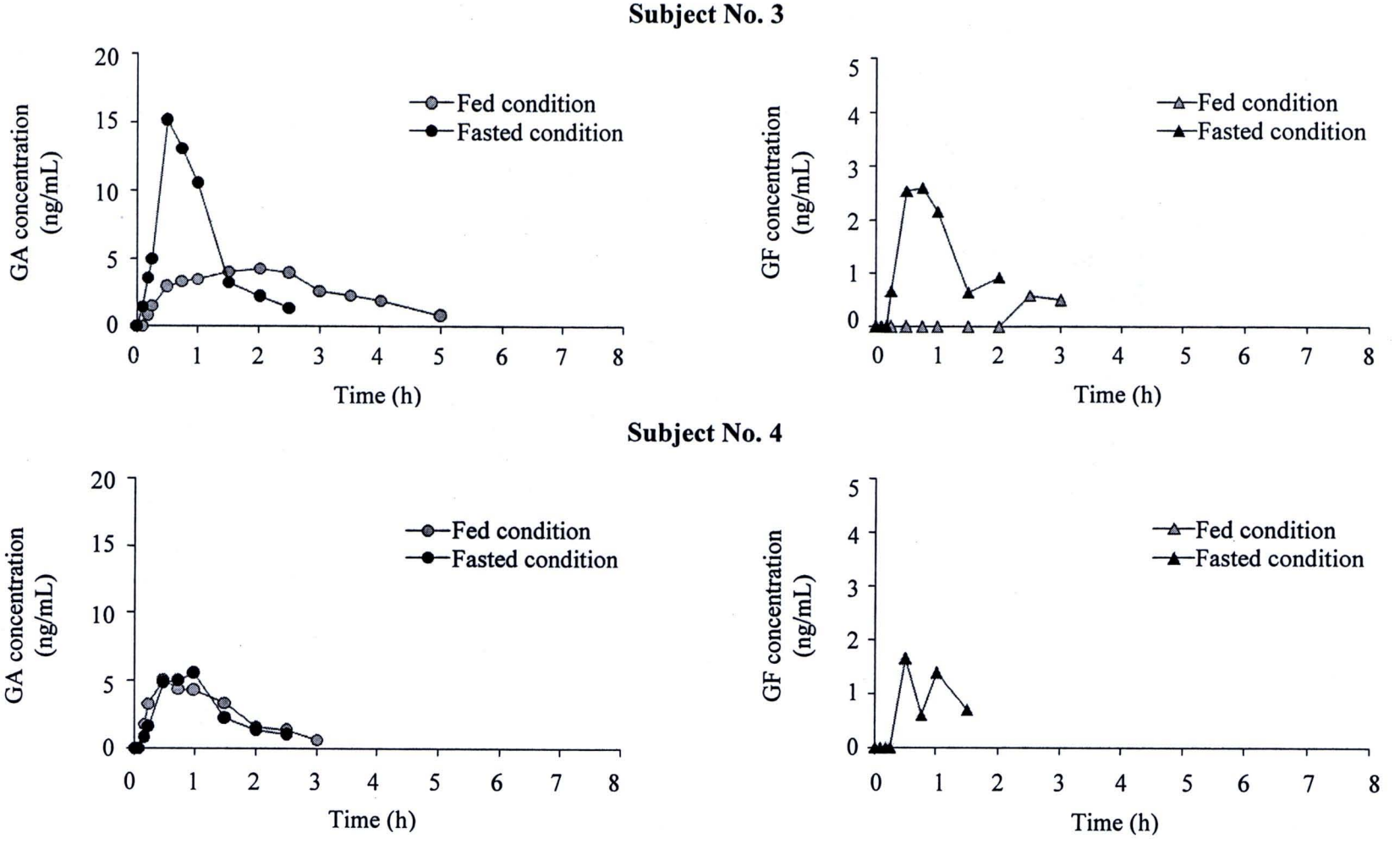

Figure 10 (Continued).

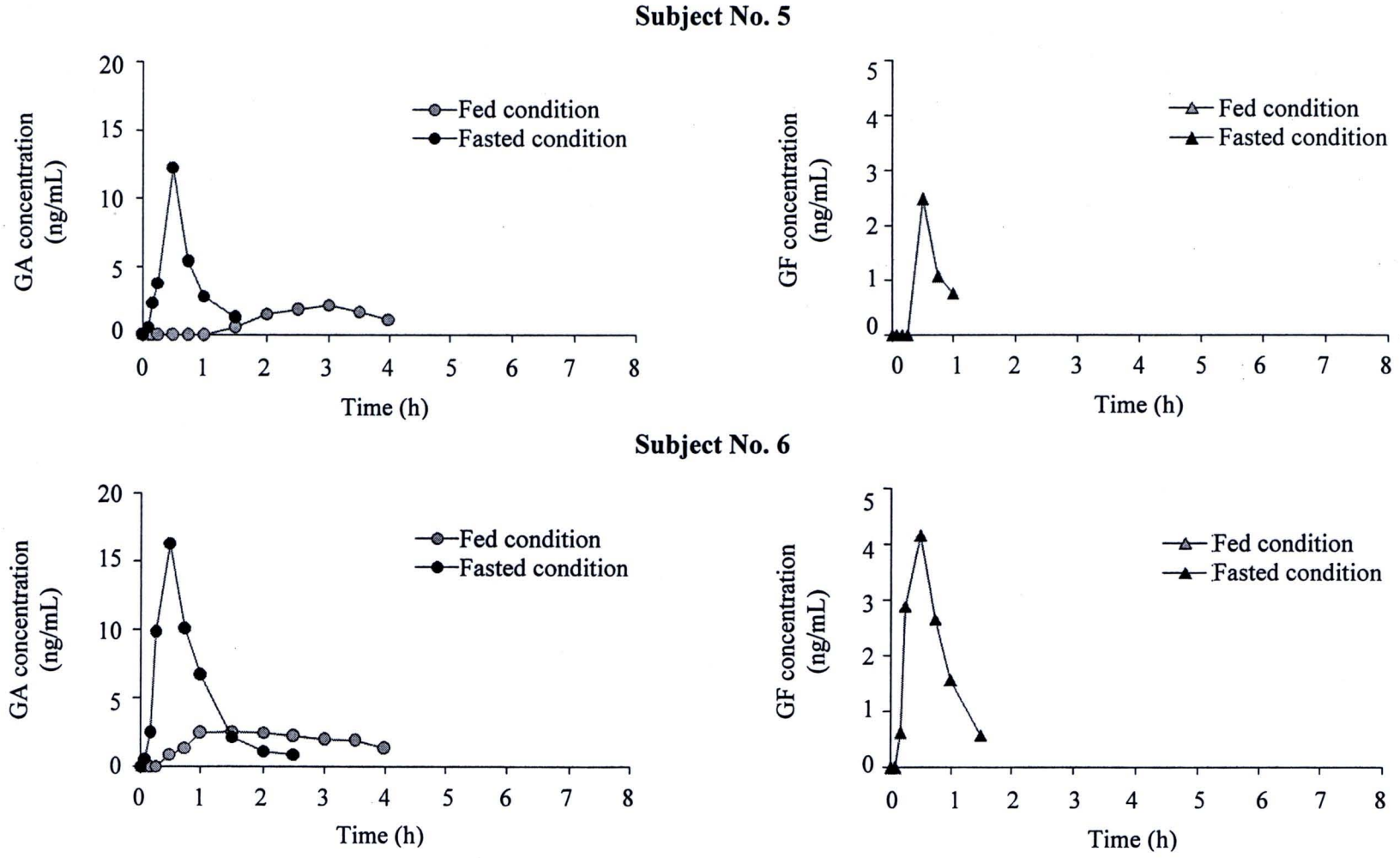

Figure 10 (Continued).

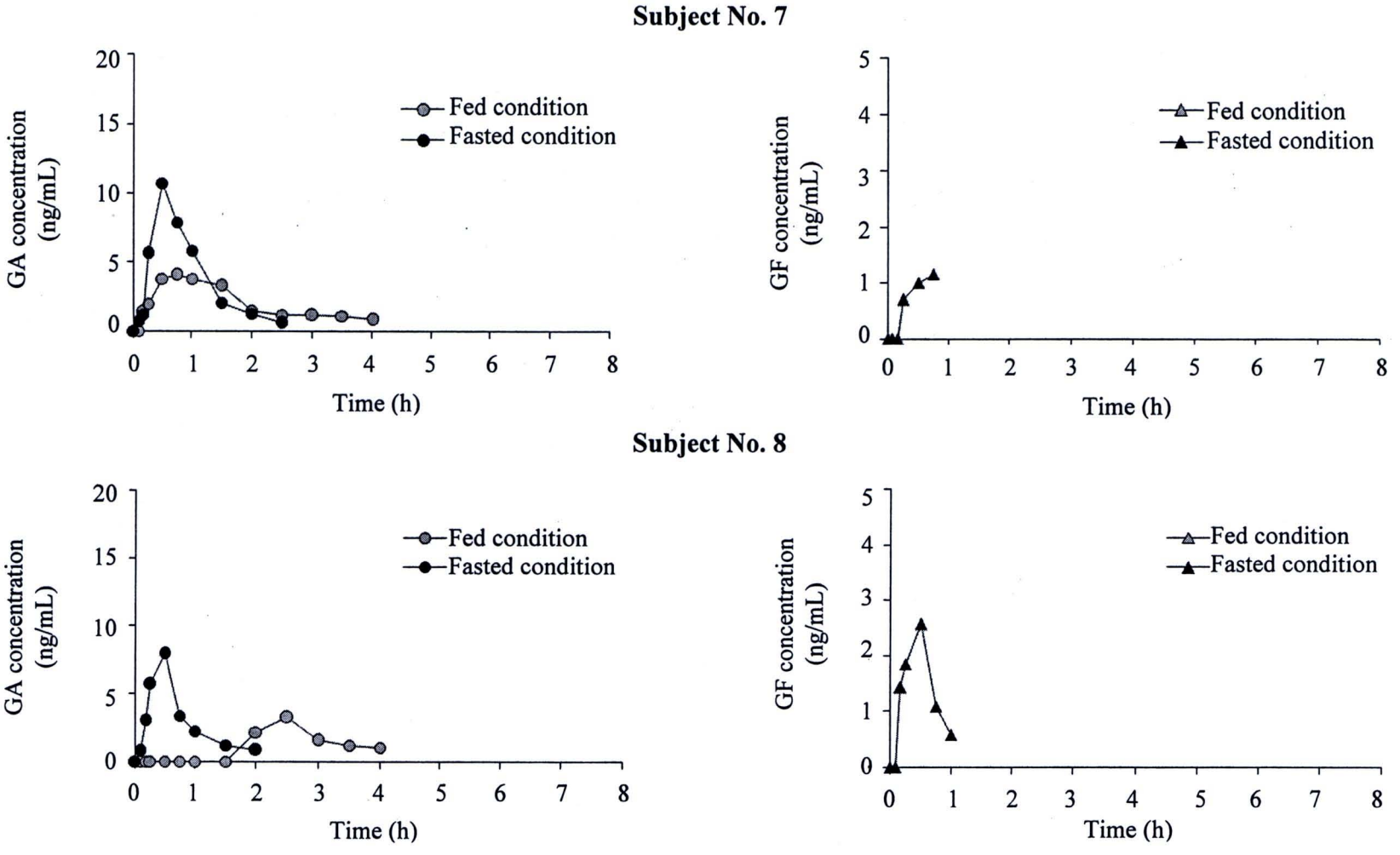

Figure 10 (Continued).

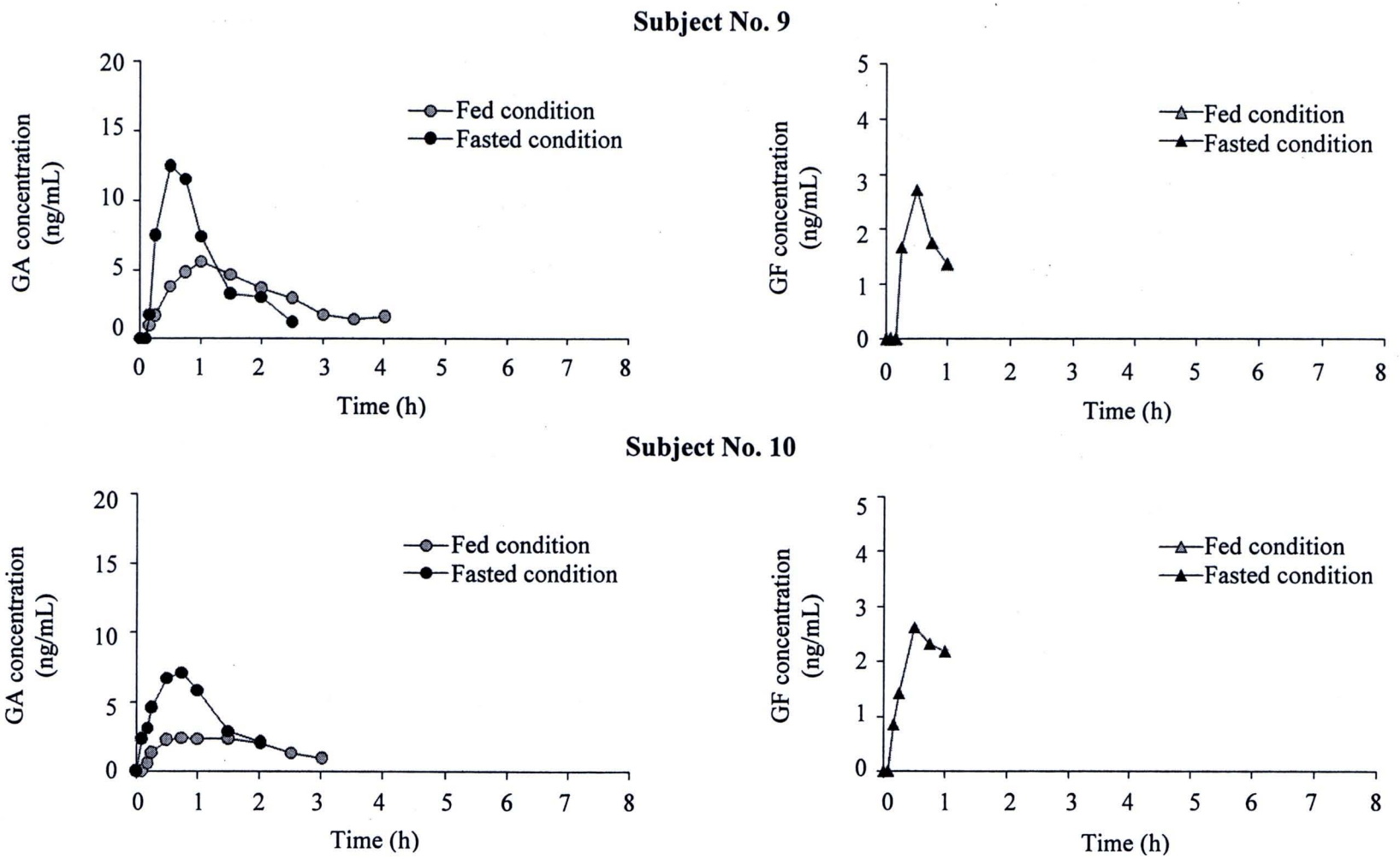

Figure 10 (Continued).

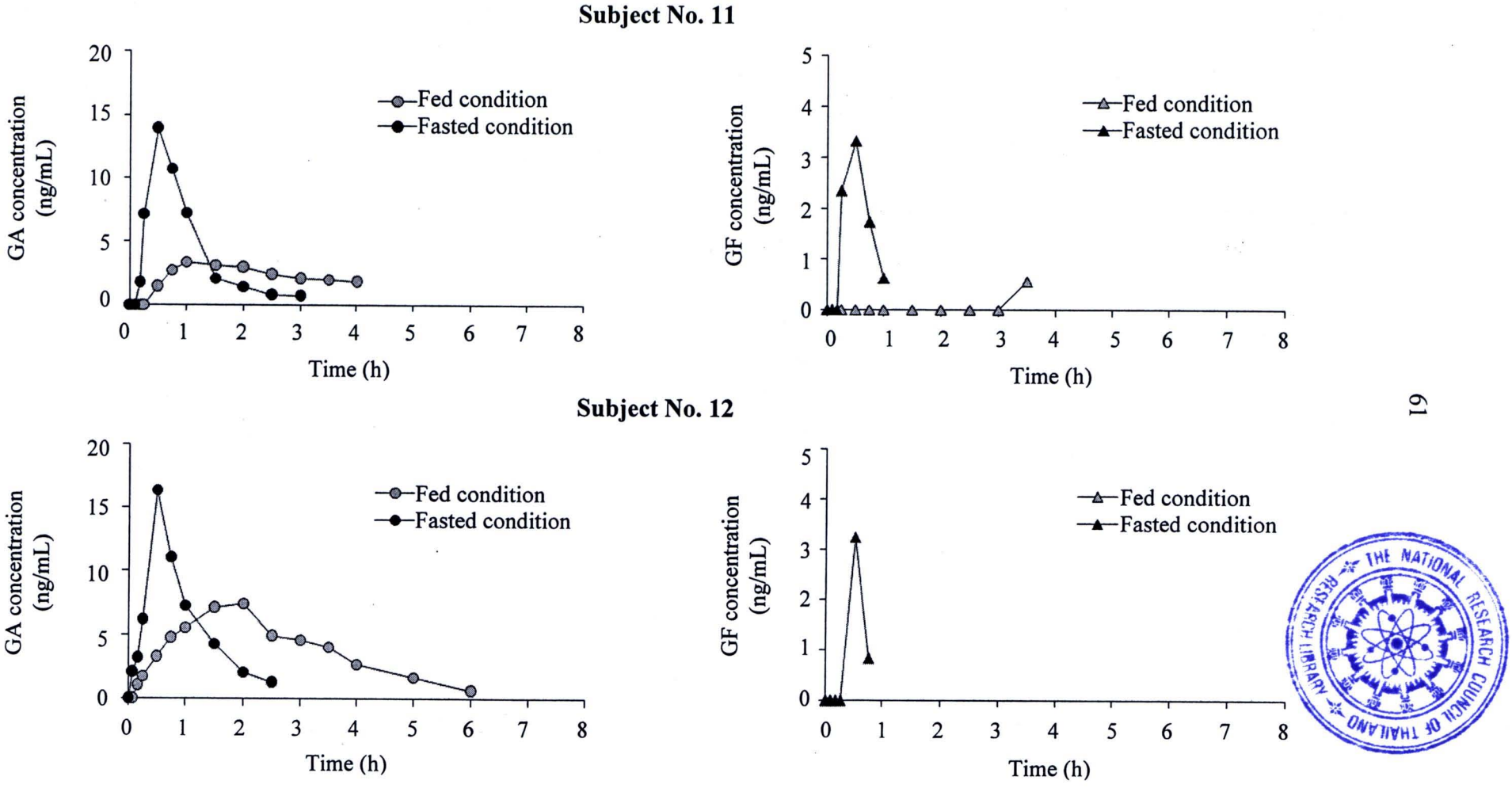

Figure 10 (Continued).

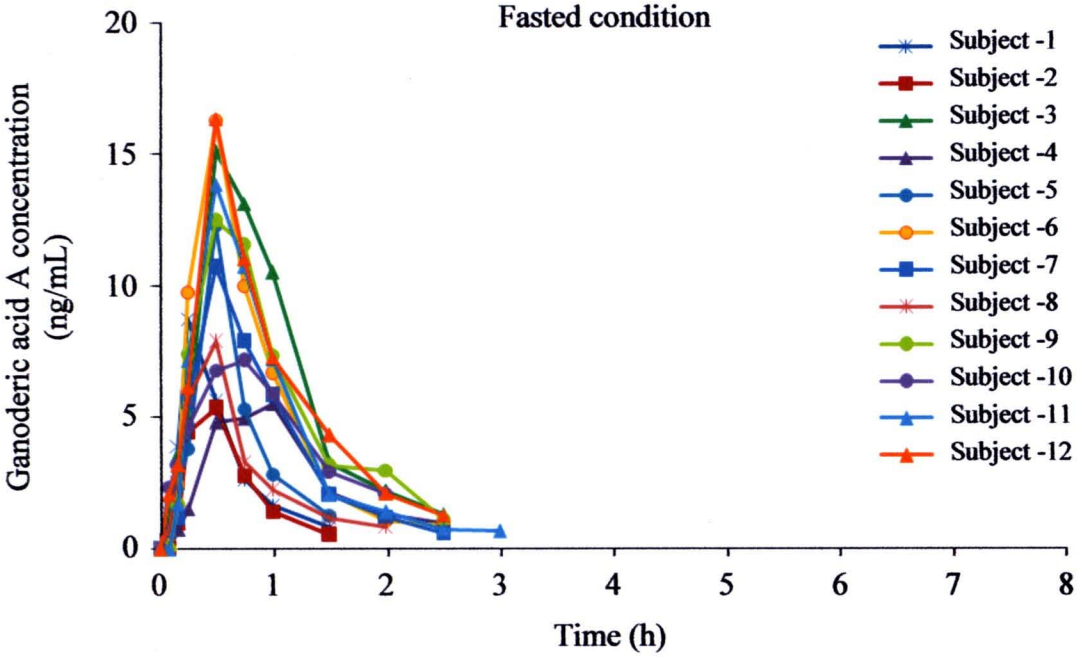

Figure 11A Composite of individual plasma ganoderic acid A concentration-time curves (n = 12) after a single oral dose of MG2FB-WE under fasted condition.

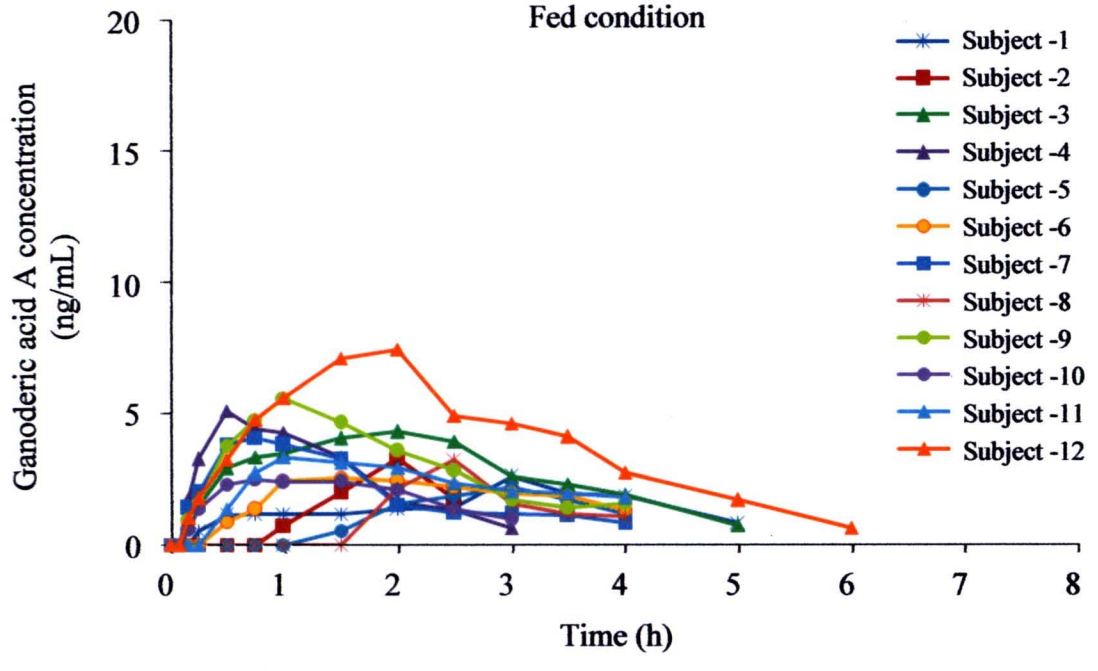

Figure 11B Composite of individual plasma ganoderic acid A concentration-time curves (n = 12) after a single oral dose of MG2FB-WE under fed condition.

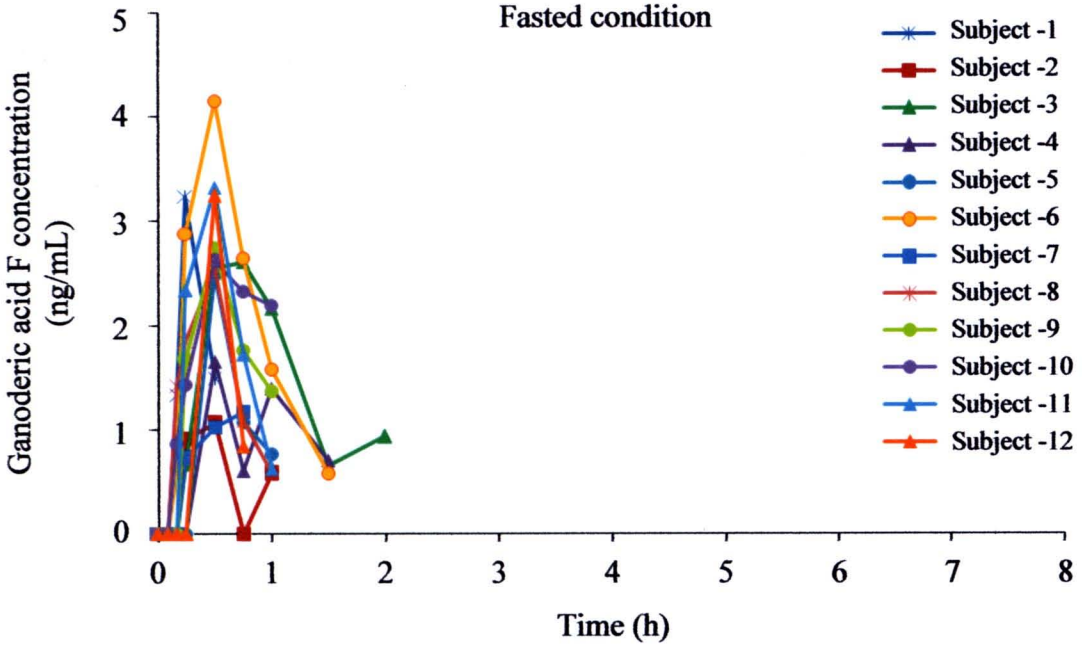

Figure 11C Composite of individual plasma ganoderic acid F concentration-time curves (n = 12) after a single oral dose of MG2FB-WE under fasted condition.

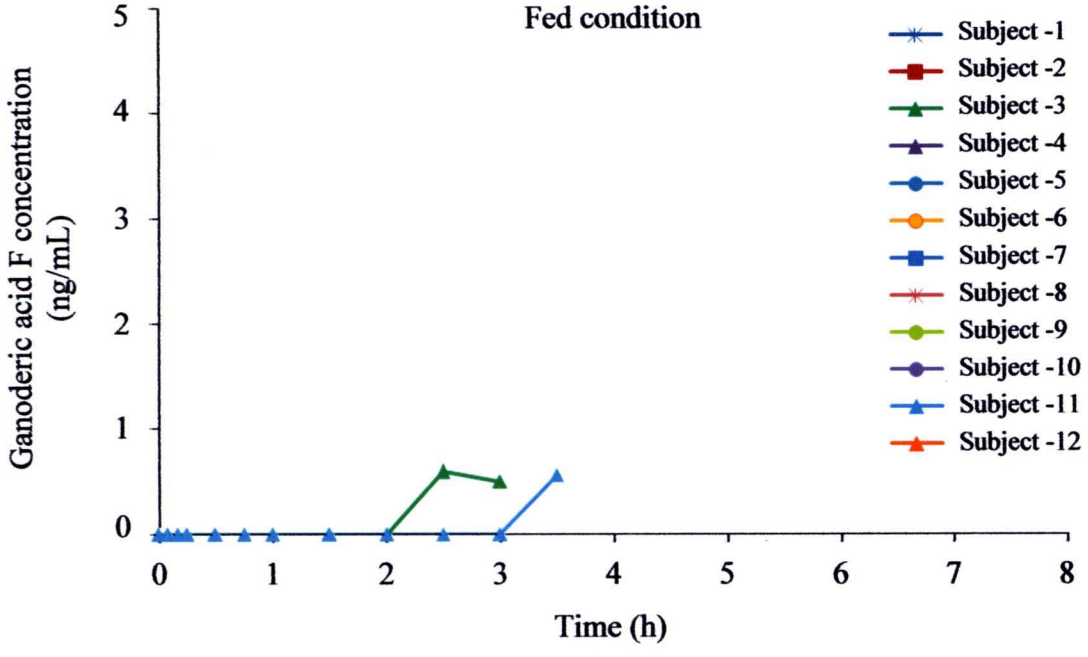

Figure 11D Composite of individual plasma ganoderic acid F concentration-time curves (n = 12) after a single oral dose of MG2FB-WE under fed condition.

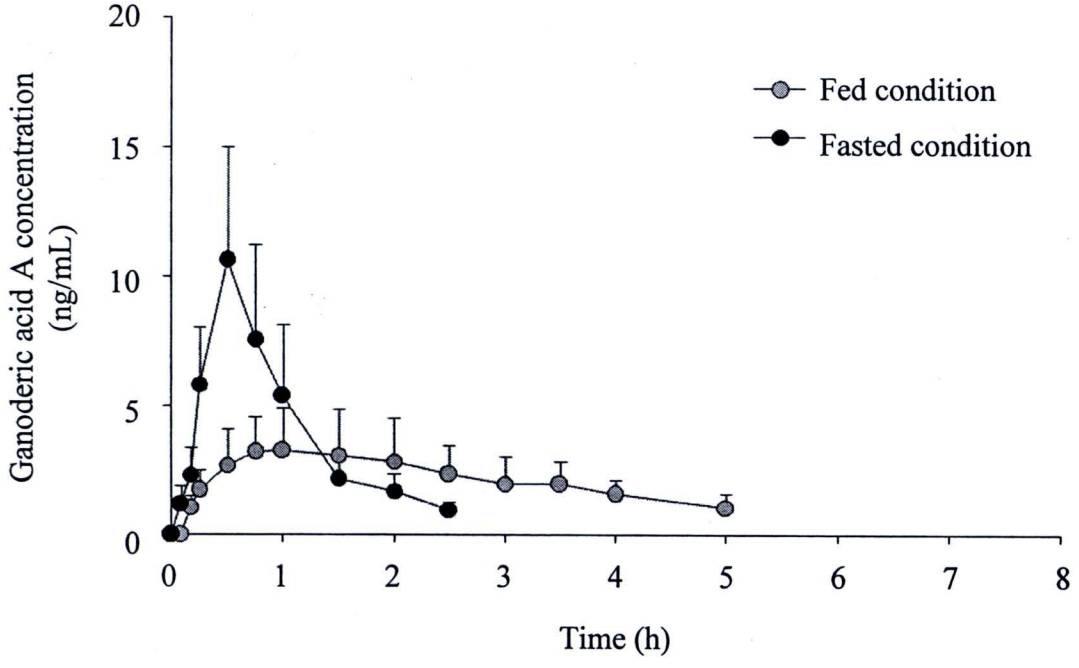

Figure 12A Mean plasma ganoderic acid A concentration-time curves after a single oral dose of MG2FB-WE under fasted or fed condition.

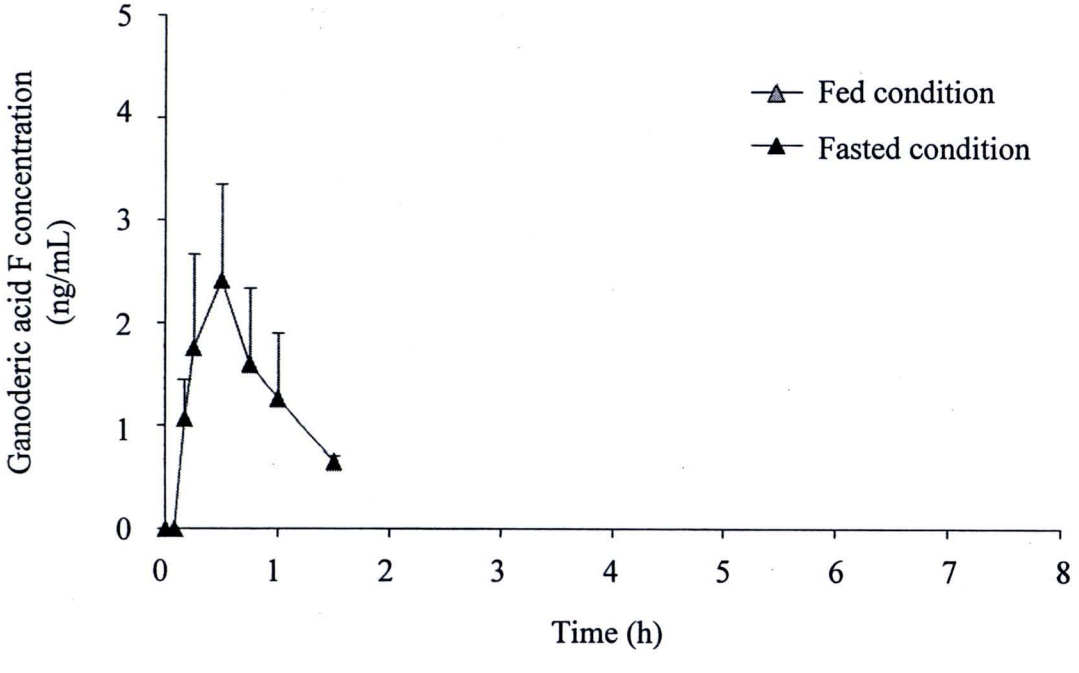

Figure 12B Mean plasma ganoderic acid F concentration-time curve after a single oral dose of MG2FB-WE under fasted condition (mean plasma ganoderic acid F concentration-time curve under fed condition is not shown due to insufficient data for calculation).

The pharmacokinetic parameters of ganoderic acids A and F (C_{max}, T_{max}, AUC_{0-8}, $AUC_{0-\infty}$ and $t_{1/2}$) following a single oral administration of MG2FB-WE under fasted or fed condition were determined and are respectively presented in Table 14A and 14B.

Mean C_{max} of ganoderic acid A decreased significantly from 10.99 ± 4.02 ng/mL under fasted condition to 3.84 ± 1.56 ng/mL under fed condition. Mean values of T_{max} between fasted and fed conditions (0.54 ± 0.18 h *versus* 1.67 ± 0.88 h) as well as $t_{1/2}$ (0.62 ± 0.17 h *versus* 1.34 ± 0.65 h) of ganoderic acid A were significantly different. Nonetheless, there was no significant difference in mean values of AUC_{0-8} (9.58 ± 4.08 *versus* 8.75 ± 5.32 ng.h/mL) and $AUC_{0-\infty}$ (10.53 ± 4.32 *versus* 11.02 ± 5.54 ng.h/mL) of ganoderic acid A between fasted and fed conditions (Table 14A).

Under fasted condition, mean C_{max} and T_{max} of ganoderic acid F were 2.57 ± 0.91 ng/mL and 0.52 ± 0.13 h, respectively. Mean values of AUC_{0-8}, $AUC_{0-\infty}$, as well as $t_{1/2}$ of ganoderic acids F under fasted condition were 1.81 ± 0.76 ng.h/mL, 2.42 ± 0.93 ng.h/mL and 0.48 ± 0.22 h, respectively (Table 14B). Since the plasma ganoderic acid F concentrations at any time points under fed condition were below the LLOQ in most of the enrolled subjects, the pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-8}, $AUC_{0-\infty}$, including $t_{1/2}$ were not be determined and therefore were not be statistically compared with those under fasted condition.

Table 14A Pharmacokinetic parameters of ganoderic acid A after a single oral administration of MG2FB-WE under fasted or fed condition

Subject No.	C_{max} (ng/mL)		T_{max} (h)		AUC_{0-8} (ng.h/mL)		$AUC_{0-\infty}$ (ng.h/mL)		$t_{1/2}$ (h)	
	Fasted condition	Fed condition	Fasted condition	Fed condition	Fasted condition	Fed condition	Fasted condition	Fed condition	Fasted condition	Fed condition
1	8.74	2.58	0.25	3.00	4.80	7.16	5.39	8.57	0.47	1.16
2	5.36	3.25	0.50	2.00	3.53	3.33	3.79	4.70	0.35	0.55
3	15.15	4.29	0.50	2.00	15.33	13.55	16.81	14.70	0.77	1.07
4	5.53	5.08	1.00	0.50	6.82	7.93	8.06	8.61	0.78	0.75
5	12.29	2.20	0.50	3.00	6.68	4.23	7.38	5.66	0.37	0.86
6	16.31	2.54	0.50	1.50	12.78	7.29	13.73	10.72	0.77	1.80
7	10.72	4.05	0.50	0.75	9.73	8.17	10.18	10.05	0.55	1.56
8	7.91	3.22	0.50	2.50	5.71	4.30	6.44	7.04	0.62	1.81
9	12.48	5.58	0.50	1.00	13.51	12.03	14.63	15.07	0.68	1.38
10	7.17	2.49	0.75	0.75	8.95	5.52	11.03	6.77	0.68	0.92
11	13.88	3.33	0.50	1.00	12.47	8.90	13.32	16.63	0.87	2.99
12	16.36	7.45	0.50	2.00	14.59	22.59	15.64	23.68	0.57	1.18
Mean	10.99**	3.84	0.54*	1.67	9.58	8.75	10.53	11.02	0.62*	1.34
SD	4.02	1.56	0.18	0.88	4.08	5.32	4.32	5.54	0.17	0.65
% CV	36.54	40.53	33.13	52.87	42.61	60.78	41.02	50.25	26.75	49.01

* $p < 0.01$, ** $p < 0.001$ denote statistically significant as compared to fed condition according to paired Student's *t*-test

Table 14B Pharmacokinetic parameters of ganoderic acid F after a single oral administration of MG2FB-WE under fasted or fed condition

Subject No.	C_{max} (ng/mL)		T_{max} (h)		AUC_{0-8} (ng.h/mL)		$AUC_{0-\infty}$ (ng.h/mL)		$t_{1/2}$ (h)	
	Fasted condition	Fed condition	Fasted condition	Fed condition	Fasted condition	Fed condition	Fasted condition	Fed condition	Fasted condition	Fed condition
1	3.24	ND	0.25	ND	ND	ND	ND	ND	ND	ND
2	1.05	ND	0.50	ND	ND	ND	ND	ND	ND	ND
3	2.61	ND	0.75	ND	2.77	ND	3.87	ND	0.82	ND
4	1.65	ND	0.50	ND	1.26	ND	1.77	ND	0.51	ND
5	2.49	ND	0.50	ND	0.99	ND	1.54	ND	0.51	ND
6	4.16	ND	0.50	ND	2.97	ND	3.26	ND	0.34	ND
7	1.17	ND	0.75	ND	ND	ND	ND	ND	ND	ND
8	2.56	ND	0.50	ND	1.41	ND	1.64	ND	0.28	ND
9	2.73	ND	0.50	ND	1.57	ND	2.94	ND	0.69	ND
10	2.62	ND	0.50	ND	ND	ND	ND	ND	ND	ND
11	3.33	ND	0.50	ND	1.73	ND	1.91	ND	0.21	ND
12	3.25	ND	0.50	ND	ND	ND	ND	ND	ND	ND
Mean	2.57	ND	0.52	ND	1.81[#]	ND	2.42[#]	ND	0.48[#]	ND
SD	0.91	ND	0.13	ND	0.76	ND	0.93	ND	0.22	ND
% CV	35.50	ND	24.72	ND	41.89	ND	38.28	ND	46.51	ND

ND = Can not be determined because the plasma ganoderic acid F concentrations at any time points were below the LLOQ in most of the enrolled subjects

[#] Pharmacokinetic parameters with ND were not taken into account