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Isolation and Preparative Purification for Ginkgolides A and B*

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Abstract In this paper, a simple preparative method for isolation and purification of ginkgolides A and B was developed. As starting material, a commercially available standardized ginkgo extract (EGb_{761}, containing 24% flavonoid and 6% terpene trilactones) was used. After a pretreatment step, optimized by the uniform design method, the concentrated intermediate extract with high content of GA and GB (+90%) was separated into the individual terpenes by preparative liquid chromatography eluted with petroleum ether-ethylacetate. Analysis of products was carried out by means of HPLC-ELSD (evaporative light-scattering detector). The results show that ginkgolides A and B are obtained in higher yield and better purity.

Keywords ginkgolide A, ginkgolide B, isolation and purification, uniform design, preparative liquid chromatography

1 INTRODUCTION

Ginkgolides A, B, C and J (further abbreviated as GA, GB, GC and GJ), terpenes with unique C_{20} cage structure[1], could specifically inhibit platelet-activating factor (PAF) binding to its receptors in various cells and tissues (see Fig. 1). Ginkgolides have been proved to be highly effective in various animal pathological models including airway hyperreactivity, shock, graft rejection, renal diseases and numerous other experimental conditions[2]. Phase Ⅲ clinical trial with these compounds has been completed[3] (burn injury and sepsis) and the first PAF antagonists are demonstrated to counteract various pathologies in men. Among these terpenes, ginkgolide B is the most potent PAF antagonist.

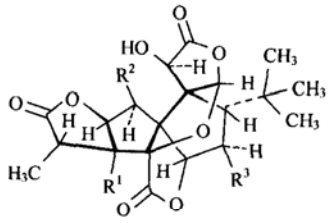

Figure 1 Structures of ginkgolides
ginkgolide A: R^1=OH, R^2=R^3=H;
ginkgolide B: R^1=R^2=OH, R^3=H;
ginkgolide C: R^1=R^2=R^3=OH;
ginkgolide J: R^1=R^3=OH, R^2=H

Because of the potential usefulness of pure GB or a mixture of ginkgolides as novel drugs against asthma and shock as well as for quality control purposes, there is a high demand for the pure compounds and the commercial availability of larger quantities in +95% purity. Although some different methods for isolation and purification of these terpenes have been developed, they still have shortcomings such as low recovery, tedious procedure and especially the difficulty in separating the pairs GA/GB and GC/GJ. According to the literature[4–6], the separation of GA and GB was only achieved by a 10—15-step fractional crystallization, repeated column chromatography, costly reversed-phase high performance liquid chromatograph(HPLC) and medium pressure liquid chromatography (MPLC) with gradient elution. Both the demand and the difficulties prompted efforts to find a more efficient method for preparing pure compound.

Solvent extraction and preparative liquid chromatography, which are different with respect to application mechanism, are two important methods to obtain ginkgolides from *Ginkgo biloba L.* leaves. In solvent extraction process, it is vital to choose the suitable solvent or solvent system, whose polarity is similar to that of the required ginkgolides. It is especially proper for preliminary fractionation work. By comparison with solvent extraction, it is the key point to choose the correct eluent and stationary phase for preparative liquid chromatography, which has an advantage of high selectivity, but requires pretreatment of material. It is believed the best results will be achieved by a combination of methods mentioned above, that is to say, first derive an enriched mixture of GA and GB by solvent extraction from commercial available standardized ginkgo extract, then separate the enriched mixture into individual compounds by preparative liquid chromatography to achieve GA and GB in high purity.

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To optimize the pretreatment process, an experimental approach by uniform design was used to characterize the various factors that affect the solvent extraction process. Fang and Wang at the Mathematical Research Institute of Chinese Academy of Science put forward a new uniform design method based on the number theory, to distribute the experimental points uniformly in the factor space[7]. The major advantage of uniform design is that when compared to commonly known methods such as factorial design, the number of experiments can be significantly reduced to produce reliable results even when the number of levels for each experimental variable is large. In addition, its corresponding Statistical Analysis Software kit makes it possible to establish accurate regression equations, and analyze the effect of each factor quantitatively. Consequently the optimal extracting conditions obtained are reliable with high confidence level.

Next, the separation by preparative liquid chromatography in which 40 mm internal diameter axial compression column was performed. Fractions containing the single compounds GA and GB were pooled and evaporated. Further purification to powders with a purity of +98% was achieved by a combination of recrystallization. Final purity was assessed by HPLC-ELSD(evaporative light-scattering detector) to confirm that GA and GB in high purity and better yield were prepared.

2 EXPERIMENTAL

2.1 Materials

Two different starting material can be used: (a) dried ginkgo leaves and (b) a commercially available standardized ginkgo extract containing 24% flavonoid glycosides and 6% terpene trilactones (ginkgolides and biloblide). Both sources have advantages and disadvantadges. In view of the condition of the laboratory, the commercial standard samples from Tianjin Jinye Purified Plant Products Factory were used. All organic solvents were of analytical grade except that MeOH and C_4H_8O (tetrahydrofuran) were of chromatographic grade.

2.2 Apparatus and analysis

The analytical HPLC system consists of a Spectra SYSTEM P4000 pump (Thermo Separation Products, Inc., USA), a ELSD500 detector (Alltech Associates, Inc., USA), and an injector (20 μl sample loop) from Rheodyne. Analyses[8] were made using SynChropak RP-P C_{18} column (250 mm×4.6 mm ID, 5 μm; MICRA Scientific, Inc., USA). The data acquisition system was ChromQuest (Ver 2.1, ThermoQuest Corporation, USA).

Mobile phase: H_2O:MeOH:C_4H_8O=75:20:10 (by volume); flow rate: 1.0 ml·min^{-1}; drift tube temperature:105℃; gas flow rate (N_2): 2.75 L·min^{-1}.

The preparative HPLC system was Lab-pre Preparative HPLC System (Gilson Inc., France) equipped with Gilson 306 pump and Gilson 206 fraction collector, ELSD500 detector, and injector (5 ml sample loop) from Rheodyne. The data acquisition system was Unipoint (Ver 1.65, Gilson Inc., France) installed on a personal computer. The size of preparative column was 300 mm×40 mm (packed with silicagel H, 10—40 μm, Tianjin Scientific Instrument Co., China). The mobile phase composition was ethylacetate-petroleum ether(8:2, by volume) at flow rate of 25 ml·min^{-1}.

2.3 Procedure

2.3.1 *Pretreatment*

Ginkgolides are soluble in ethyl acetate, petroleum ether, benzene, methanol and dichloromethane which may be used as the extraction solvents. For the aim of gaining the intermediate extract (mixture of ginkgolides) with high content of GA and GB, four pretreatment schemes[5,6] were designed with different combination of solvents and different extracting procedure. With the use of HPLC-ELSD, the extracts obtained by four schemes were analyzed respectively. The results of three schemes were out of satisfaction. Therefore, we sketchily list their procedures in Table 1 and detail the better one later.

Table 1 shows the very low yield of total ginkgolides in these schemes. To induce phase separation of aqueous solution and preconcentration of extracted ginkgolides into organic phase, an appropriate amount of NaCl was used in Scheme 4 described below.

Approximately 40 g of standardized ginkgo extract were dissolved in certain quantity petroleum ether. After adding water and 10 min of stirring, the solution was filtered through a Buchner funnel and the filtrate was transferred into a 500 ml separating funnel. There formed a clear interface between two phases. The aqueous phase was separated and then extracted with petroleum ether twice at room temperature, thereby getting rid of remaining lipidic substance. After addition of NaCl (10 g in 100 ml H_2O), the aqueous phase was extracted with EtOAc five times at room temperature. The Na_2CO_3 solution (1 mol·L^{-1}) was added in the organic layer obtained above, since flavonoids react with the alkaline solution to form carbonate which can be dissolved in the aqueous solution. After drying with anhydrous Na_2SO_4 and evaporation in vacuum, 689 mg straw yellow mass resulted that contained approximately 88% GA and GB. The yield of total ginkgolides is 25.3%, which is better than the results shown in Table 1, so the further studies were made.

Table 1 The procedure and results of three pretreatment schemes

	Scheme 1	Scheme 2	Scheme 3
Step 1	dissolved in 70% aqueous methanol at 70℃ and filtered	dissolved in 70% aqueous acetone at 80℃ and filtered	dissolved in ethyl acetate and filtered then evaporating the filtrate
Step 2	extracting the filtrate with tetra-chloromethane	extracting the filtrate with tetra-chloromethane	dissolved in 30% aqueous methanol and filtered
Step 3	adjusting pH=8.5 for aqueous layer with NaOH solution and extracting with benzene	adjusting pH=7.5 for aqueous layer with NaOH solution and extracting with ethyl acetate	extracting the filtrate with tetra- chloromethane
Step 4	drying organic layer with anhydrous $(NH_4)_2SO_4$ then by evaporating, A1 was obtained	drying organic layer with anhydrous Na_2SO_4 then by evaporating, B1 was obtained	same as Step 3 and Step 4 in scheme 2 then C1 was obtained
Step 5	adjusting pH=2.0 for aqueous layer obtained in Step 3	adjusting pH=3.5 for aqueous layer obtained in Step 3	same as Step 5 in Scheme 2 except pH=2.5
Step 6	extracting with dichloromethane	extracting with diethyl ether	extracting with trichloromethane
Step 7	repeating Step 4 and A2 was obtained	repeating Step 4 and B2 was obtained	repeating Step 4 and C2 was obtained
Step 8	dissolving A1 and A2 in acetone	dissolving B1 and B2 in ethanol	dissolving C1 and C2 in ethanol
Step 9	adding plumbous acetate in solution then filtering and evaporating to get product	adding activated charcoal in solution then filtering and evaporating to get product	adding plumbous acetate in solution then filtering and evaporating to get product
material, g	40	40	40
total ginkgolide, mg	41	24	103
yield of total ginkgolides, %	1.7	1.0	4.29

Note: A1, A2, B1, B2, C1 and C2 refer to different extract in schemes.

Table 2 Experimental factors and levels

Factor	Level									
	1	2	3	4	5	6	7	8	9	10
NaCl, g	10	20	30	40	50	60	70	80	90	100
EtOAc, ml	100	150	200	250	300	100	150	200	250	300
Na_2CO_3 solution, ml	5	10	15	20	25	30	35	40	45	50

Note: the dosage of NaCl in 100 ml H_2O.

Table 3 Uniform design table $[U_{10}(10^3)]$: experimental conditions and yield values

Trial No.	Condition			Experimental value of yield				
	NaCl	EtOAc	Na_2CO_3	Total ginkgolides	GA		GB	
	g	ml	ml	g	g	%	g	%
1	10	300	35	0.4243	0.204	48.1	0.163	38.45
2	20	300	15	0.5494	0.150	27.32	0.118	21.43
3	30	250	50	1.1742	0.491	41.78	0.342	29.16
4	40	250	30	0.5263	0.212	40.31	0.169	32.05
5	50	200	10	1.1502	0.379	32.99	0.294	25.61
6	60	200	45	1.0021	0.393	39.25	0.293	29.21
7	70	150	25	0.4757	0.182	38.35	0.112	23.45
8	80	150	5	1.5075	0.332	22.05	0.128	8.49
9	90	100	40	0.2259	0.073	32.34	0.130	57.68
10	100	100	20	0.2360	0.075	31.59	0.050	21.31

Note: the dosage of NaCl in 100 ml H_2O.

To enhance the yield of total ginkgolides, uniform design was employed to optimize the extracting conditions in pretreatment, including three key factors: the dosage of NaCl, the dosage of EtOAc and the dosage of Na_2CO_3 solution. Table 2 shows the corresponding 10 levels of each factor. Uniform design table for experiments with 3 factors are shown in Table 3[9].

2.4.2 *Preparative liquid chromatography separation*

The concentrated intermediate extract obtained in the pretreatment step was dissolved in the mobile phase of preparative liquid chromatography. Then the resulted clear light brown solution is directly injected by means of an injector with a 5 ml loop. The eluent was controlled by Unipoint software on a computer.

Fractions were collected with a fraction collector. In order to have on-line detection, a split-flow valve was installed between column and detector in this experiment. 1 ml·min^{-1} eluent into the ELSD detector was made by adjusting the split-flow valve.

Final purification of pooled preparative liquid chromatography fractions containing only individual ginkgolide was carried out by recrystallization. GA and GB were recrystallized from MeOH-H_2O=6:4 and MeOH-H_2O=4:6 respectively. Firstly the fractions were evaporated on a rotary vacuum evaporator to remove the solvents, and then the powder was dissolved in a sufficient quantity of the appropriate solvent mixture at 50℃. The solution was left in the refrigerator overnight and GA and GB were finally obtained as white crystals.

3 RESULTS AND DISCUSSION

3.1 Optimization of pretreatment using uniform design[10]

To the best of our knowledge, the uniform design method has never been reported for extracting ginkgolides from standardized ginkgo extract.

Table 3 shows the design of experiments and the responding results obtained. By conducting 10 individual experiments, the optimal extracting conditions were determined for three different factors: the dosage of NaCl (x_1), the dosage of EtOAc (x_2) and the dosage of Na_2CO_3 solution (x_3). The experimental values investigated in this work including the mass of total ginkgolides (Y_1, g) and the mass of GA (Y_2, g), and the mass of GB (Y_3, g) which are also shown in Table 3.

Then the Statistical Analysis Software kit was used to establish regression equations and find the optimal extracting conditions for gaining high mass of total ginkgolides with high content of GA and GB.

Using the following second-order polynomial model with the forward regression method for selection of variables

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{1 \leqslant i \leqslant j \leqslant 3} \beta_{ij} x_i x_j + e \qquad (1)$$

The following models are recommended

$$Y_1 = 2.43101 - 0.14122x_3 + 0.00252x_3^2 \qquad (2)$$

with $R = 87.5\%$, $S = 0.292$, $F = 11.45 > F_{0.05}(3,6) = 4.76$,

$$Y_2 = 1.47584 - 0.0915x_3 - 0.00006x_1^2 - 0.00003x_1x_2 + 0.00040x_1x_3 + 0.00021x_2x_3 + 0.00052x_3^2 \qquad (3)$$

with $R = 99.9\%$, $S = 0.012$, $F = 200.66 > F_{0.05}(7,2) = 19.35$,

$$Y_3 = 0.45263 - 0.00004x_1^2 + 0.00001x_2x_3 \qquad (4)$$

with $R = 85.1\%$, $S = 0.069$, $F = 5.816 > F_{0.05}(4,5) = 5.19$.

All these F-tests are significant at $\alpha = 0.05$, so the models are acceptable.

The "best" combination of factor-levels from the models [Eq. (2), Eq. (3) and Eq. (4)], or the optimal extracting conditions, were obtained as follows: $x_1 = 60$, $x_2 = 100$, $x_3 = 15$. Some additional experiments are necessary for confirming the optimal condition. The experimental results are shown in Table 4, which indicates the average values are close to the prediction respectively.

The results of pretreatment process of GA and GB by means of solvent extraction illustrated that this method has 90% selectivity to GA and GB, which is favorable to control the quality of drug. And the yield of total ginkgolides reaches 36.2% on average. While the recent literature[11] reported that the content of GA, GB and GC in total gingolides was above 90% with 28.1% yield. Meanwhile, no literature has reported the method about merely extracting the mixture of GA and GB from the standard ginkgo extract.

3.2 Preparative liquid chromatography separation

Figure 2 illustrated the preparative liquid chromatography separation results, a base-line separation of GA and GB was observed. By the fraction collector, individual ginkgolide solution could be available.

Final purification for GA, GB obtained in preparative liquid chromatography separation was completed by recrystallization. GA was recrystalized from MeOH-H_2O=6:4, while MeOH-H_2O=4:6 was suitable

Table 4 The experimental results at the optimal extracting condition

No.	Starting material mass	total ginkgolides	Yield of total ginkgolides	GA		GB	
	g	y_1, g	%	y_2, g	%	y_3, g	%
1	40	0.854	35.6	0.468	54.8	0.300	35.2
2	40	0.898	37.4	0.494	55.0	0.307	34.1
3	40	0.872	36.3	0.489	56.0	0.305	35.0
4	40	0.849	35.4	0.467	54.9	0.299	35.1
average		0.868	36.2	0.479	55.2	0.302	34.9
prediction		0.880		0.499		0.324	

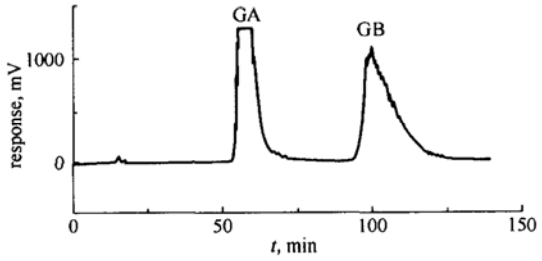

Figure 2 Chromatogram of preparative liquid chromatography separation
column: silicagel H, 10—40 μm, 300 mm×40 mm ID
mobile phase: ethylacetate: petroleum ether=8:2(by volume);
flow rate: 25 ml·min^{-1}

for GB recrystallization. When 86.2 mg enriched intermediate extract (contained 35% GB and 56% GA) dissolved in 5 ml mobile phase was applied on preparative liquid chromatography column, 30.4 mg GB and 45.2 mg GA in +95% purity could be gained. The yield of GA and GB are both above 90%.

By recrystallization, the purity of GA and GB reached +98% with 90% yield on average.

4 CONCLUSIONS

In this paper, the preparative liquid chromatography separation combined with solvent extraction is proven to be the feasible and effective way for isolation and purification for GA and GB from the standard ginkgo extract.

Uniform design was employed in the extracting process to find the optimal conditions, at which the concentrated intermediate extract of GA and GB (+90%) was gained. The enriched mixture was separated into individual compounds by preparative liquid chromatography to achieve GA and GB in +95% purity. Finally by recrystallization, the purity of GA and GB reached +98%.

NOMENCLATURE

e	random error
F	F statistical value
R	related coefficient
S	residual standard error
t	time, min
x_1	dosage of NaCl in 100 ml H_2O, g
x_2	dosage of EtOAc, ml
x_3	dosage of 1 mol·L^{-1} Na_2CO_3 solution, ml
Y	predicted value
Y_1	predicted value of the yield of total ginkgolides, g
Y_2	predicted value of the yield of GA, g
Y_3	predicted value of the yield of GB, g
y_1	experimental value of the yield of total ginkgolides, g
y_2	experimental value of the yield of GA, g
y_3	experimental value of the yield of GB, g
α	test level
β_0	regression coefficient
β_i	regression coefficient
β_{ij}	regression coefficient

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