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Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 811–817

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Analysis of ginkgolides and bilobalide in *Ginkgo biloba* L. extract injections by high-performance liquid chromatography with evaporative light scattering detection

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Received 17 February 2003; received in revised form 20 May 2003; accepted 22 May 2003

Abstract

A RP-HPLC method with evaporative light scattering detection (ELSD) was developed for the determination of ginkgolides and bilobalide in *Ginkgo biloba* L. extract injections. The samples were extracted with ethyl acetate and the resulting extract was purified by aluminum oxide column. The resultant solution was determined by HPLC on a C_{18} column with methanol–water (33:67, v/v) as eluent. The optimum ELSD parameters were set. The recovery of the method was between 98.3 and 102.1%. The method is suitable for routine quantitation of terpenes in *Ginkgo biloba* L. extract injections.
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Keywords: Ginkgolides; Bilobalide; *Ginkgo biloba* L. extract injection; HPLC determination

1. Introduction

In recent years, many phytopharmaceuticals containing *Ginkgo biloba* L. extract (GBE), such as tablets, capsules and injections, have been extensively used to treat cerebrovascular and peripheral circulatory disorders of the elderly and to cure asthma [1]. The stable GBE injections were researched and developed in our laboratory. The pharmacological results demonstrate that GBE injections have the following functions: scavenging free radicals, regulating the circulatory system, improving hemorheology, selectively inhibiting the platelet-activating factor (PAF) and protecting the tissue. It is also employed in the treatment of cerebral and peripheral circulatory disturbances, and can relieve the clinical symptoms more effectively and quickly. The pharmacological efficacy of GBE injections is mainly due to the diterpenes, ginkgolides A, B and C (GA, GB and GC), and the sesquiterpene, bilobalide (BB; Fig. 1) [2], and various flavonoids may also play an important role. Therefore, the contents of ginkgolides and BB should be accurately determined in order to control the quantity of GBE injections.

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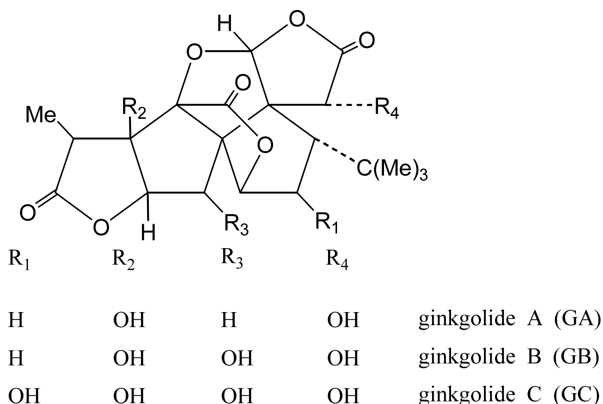
doi:10.1016/S0731-7085(03)00309-1



R_1	R_2	R_3	R_4	
H	OH	H	OH	ginkgolide A (GA)
H	OH	OH	OH	ginkgolide B (GB)
OH	OH	OH	OH	ginkgolide C (GC)

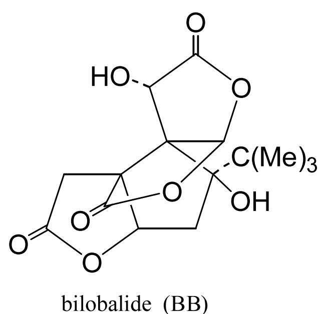


Fig. 1. The chemical structures of the compounds studied.

Many methods have been tried to analyze ginkgolides and bilobalide. In the method using initial high-performance liquid chromatography (HPLC) with ultraviolet (UV) [3], many trace impurities interfere because of the poor UV characteristics of terpenes and their low concentrations in GBE and phytopharmaceuticals. Several investigators have applied refractive index as an alternative detection method [3,4]. Although this method is more suitable and has been used with considerable success, sensitivity and baseline stability still remain a problem. Other techniques including gas chromatography–flame ionization detection after silylation [5], HPLC–mass spectrometry [6] and NMR [7] have been proposed and are used. However, these three methods are not suitable for routine determination in terms of cost, time or complex sample preparation.

Evaporative light scattering detection (ELSD) is a mass and non-selective detector that responds to the number and size of nonvolatile particles. Therefore, it is applied to the analysis of poor UV absorption and nonvolatile compounds. In 1995, Camponovo et al. [8] reported the coupling of HPLC with ELSD in order to detect ginkgolides and BB. Results indicate that it is one of the most suitable methods for routine determinations of such compounds.

This paper describes a rapid, simple, and reliable method to analyze GA, GB, GC, and BB in GBE injections by HPLC-ELSD.

2. Experimental

2.1. Materials

GBE were purchased from Xinyi Bailuda Pharmaceutical Ltd. Co. GBE injections were prepared in our laboratory (Lot: 990401, 990402, and 990403). GA, GB, GC, and BB standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of the standards was checked by TLC, HPLC, and NMR. Methanol and acetonitrile (Merck, Darmstadt, Germany) were of HPLC quality. Acetone, ethyl acetate, tetrahydrofuran, and hydrochloric acid were of analytical reagent grade. The water needed in the experiment was double distilled. The solid-phase extraction column packed with neutral aluminum oxide (0.5 g) for chromatographic use was prepared in our laboratory.

2.2. Chromatographic conditions

The HPLC system was composed of a LC-10ATvp pump (Shimadzu, Kyoto, Japan), an evaporative light scattering detector (Sedex 75, Sedere, Alfortville, France), a Phenomenex model 7725i injector valve with 20 μl loop (Torrance, CA), and an HS2000 series of chromatographic workstation (Hangzhou Yingpu Co., Hangzhou, China).

The columns used for the separation of ginkgolides and BB were Diamonsil C_{18} (250 mm × 4.0 mm ID, 5 μm; Dikma, Beijing, China) and YWG C_{18} (250 mm × 4.0 mm ID, 10 μm; National Chromatography R&A Center, Dalian, China). The mobile phase was methanol–water (33:67, v/v)

at a flow rate of 1.0 ml/min. ELSD conditions were optimized in order to achieve maximum sensitivity: temperature of the nebulizer was at 40 °C, and the air used as the nebulizing gas was at a pressure of 3.5 bar.

2.3. Standard solutions

2.3.1. Preparation of stock standard solution

Ginkgolide and BB stock solution were prepared with the concentration of 2 mg/ml BB, 1.5 mg/ml GC, 2 mg/ml GA, and 1.5 mg/ml GB in methanol, and the resultant solution was kept in a freezer.

2.3.2. Preparation of standard solutions

BB (100, 250, 400, 600, 800 μg/ml), GC (75, 187.5, 300, 450, 600 μg/ml), GA (100, 250, 400, 600, 800 μg/ml), and GB (75, 187.5, 300, 450, 600 μg/ml) standard solutions were prepared by the dilution of the stock standard solution with methanol.

2.4. Formulation of the injections

Each ampoule contains 17.5 mg GBE. GBE injections were developed for clinical trial.

2.5. Sample preparation

GBE injections (10 ml) were taken for the separation and determination of ginkgolides and BB. Two drops of 2% hydrochloric acid solution were added into the sample solution and mixed well. The resultant solution was extracted four times with 15, 10, 10, and 10 ml ethyl acetate, respectively, each time shaking for 1 min. The organic phase was combined and evaporated to dryness. The residue was dissolved in 5 ml of 70% aqueous acetone and transferred to the solid-phase extraction column that was first washed with 35 ml of 70% aqueous acetone. Then, the column was washed with 40 ml acetone. After evaporation of the eluent under reduced pressure, the residue was dissolved in 5 ml methanol, and 20 μl of the resultant solution was subjected to HPLC analysis. Both the samples and the standards were filtered through a 0.45-μm membrane filter before injection.

3. Results and discussion

3.1. Optimization of the chromatographic system

To effectively separate the terpenes in GBE injections, various mobile phase systems were investigated. GA and GB nearly coelute when acetonitrile–water was examined; propyl alcohol–tetrahydrofuran–water and methanol–water were used as eluent, which gave a better resolution of ginkgolides and BB. According to the mild operation parameters of ELSD and the applicable conditions, methanol–water was selected as eluent.

In ELSD, the nebulizer-gas pressure and drift tube temperature are the major instrumental parameters affecting the signal response. In general, large droplets are formed at low gas pressure, which results in spikes and noisy signals. On the other hand, increasing the gas pressure results in a marked decrease of signal response. The optimum nebulizer-gas pressure in this case was set at 3.5 bar. With respect to the drift tube temperature, solvent evaporation is not completed at low temperature and the detector response is decreased at high temperature. Therefore, the optimal drift tube temperature was determined to be 40 °C.

Two kinds of C_{18} column, Diamonsil C_{18} and YWG C_{18}, were used. A typical chromatogram of GBE injections on the Diamonsil C_{18} column is shown in Fig. 2, in which a good resolution of BB, GC, GA, and GB was obtained with retention times of 13.1, 16.6, 34.6, and 39.8 min, respectively. The result was in accordance to that obtained by Camponovo et al. In Fig. 2, an unknown independent peak between BB and GC corresponded to the peak of ginkgolide J (GJ) in van Beek's chromatogram [3]. Without GJ standard, the peak could not be identified. As shown in Fig. 3, BB, GC as well as the peak at 14.6 min could not be separated completely on YWG C_{18} column and all the peaks were asymmetric, which may be due to the differences between the specificity of two kinds of packing. Additionally, the

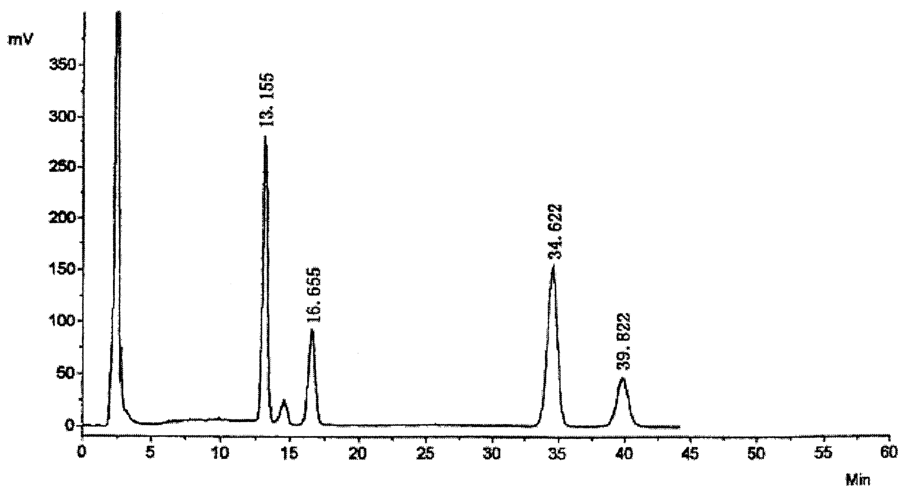


Fig. 2. Chromatogram of GBE injection on a Diamonsil C_{18} column.

composition of the eluent had to be adjusted according to the column used.

3.2. Calibration

According to the quantitative principles of ELSD, detector response is given by $y = ax^b$, where y is the peak area, x is the sample amount, and a and b are numerical coefficients. Therefore, a plot of peak area versus sample concentration is not linear, but the plot of peak area versus sample concentration in double logarithmic is linear. Calibration was performed with five different standard solutions of external standards. Linear ranges and correlation coefficient for each terpene are given in Table 1.

3.3. Sample preparation

In the method of Camponovo et al. [8], samples were prepared with and without previous cartridge purification. With the cleanup procedure, it required tedious pre-purification processes and specific separation columns. Therefore, this method

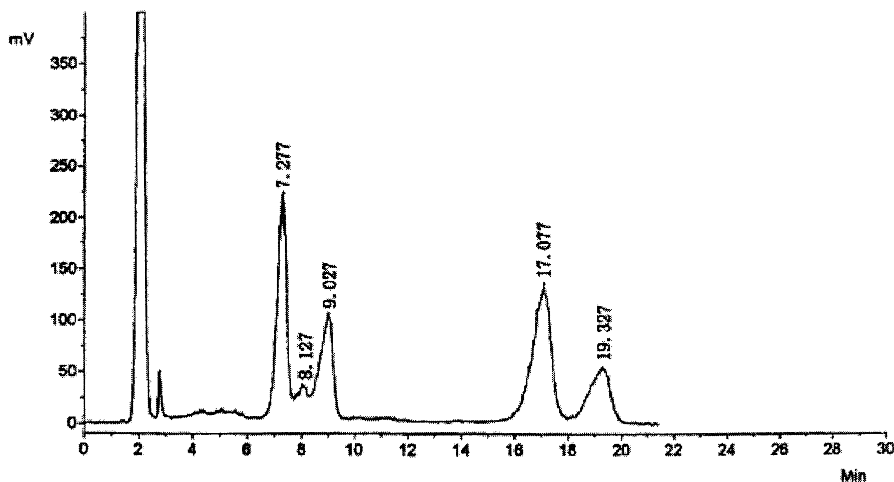


Fig. 3. Chromatogram of GBE injection on a YWG C_{18} column.

Table 1
Linear ranges and correlation coefficients (r)

Terpene	Linear ranges (μg/ml)	Correlation coefficient
BB	100–800	0.9992
GC	75–600	0.9992
GA	100–800	0.9995
GB	75–600	0.9996

was complex, time-consuming, and difficult to reproduce. Without the cleanup procedure, the chromatogram showed more peaks and higher baseline noise. Moreover, it was a disadvantage to analyze the sample that there were several peaks after the peak of GB, which is the last compound of interest.

In this HPLC-ELSD method, the addition of two drops of 2% hydrochloric acid aqueous can increase the extraction efficiency and reduce the extract time. As a result of the interferent of the neutral aluminum oxide for chromatographic use, the pre-wash step was essential. The solid-phase extraction column removed most of the flavonoids and the additives of GBE injections. To avoid the decomposition of BB caused by the alkalinity of aluminum oxide, the neutral aluminum oxide for chromatographic use was applied with small quantity, and the process of elute was rapid. Following the above procedure, the resultant solution was nearly transparent and contained less colored impurities. Since the more simple and time-saving purification method used overcame the difficulties due to background interferences from the complex matrix and the excipients in GBE injections, the chromatogram similar to that explained by Camponovo et al. [8] with the cleanup procedure was obtained. There were only five peaks of the terpenes with a quite successful resolution, which was effective for quantitative analysis of BB, GC, GA, and GB in GBE injections. Moreover, the isocratic chromatography method that was convenient and beneficial to the routine analysis in the quality control of products was applied. In order to determine the recovery of the purification procedure, the known amount of BB, GC, GA, and GB standard solutions were added into the samples, and the resultant solutions were analyzed by the above procedure (five replicates). The results are shown in Table 2. The recovery was between 98.3 and 102.1% and RSD was between 1.7 and 3.3%. Therefore, this purification procedure can provide high recovery and accuracy and was found to be acceptable for routine analysis.

3.4. Precision

The precision of injection was demonstrated by replicate injections of the sample solution. RSD was between 0.4 and 1.7%. There was no interfering peak in the chromatogram of the sample (Fig. 2).

Intra- and inter-day precisions were determined by analysis of the average amount of BB, GC, GA, and GB in an ampoule. During this period, the solution was stored under 4 °C and wrapped with aluminum foil. RSD values for intra- and inter-day precision ranged from 2.0 to 3.8 and 1.1 to 2.5%, respectively (Table 3).

3.5. Quantitative analysis of BB, GC, GA, and GB in GBE injections

Quality control is very important in the identification and characterization of preparations. The above results implied the possibility for separating

Table 2
Recovery of the sample purification

Sample	Amount of standard added (mg)	Recovered amounts (mg)					Mean recovery (%)	RSD (%)
BB	1.030	1.053	1.062	1.064	1.007	1.074	102.1	2.5
GC	1.222	1.228	1.210	1.253	1.206	1.242	100.4	1.7
GA	1.561	1.553	1.531	1.488	1.542	1.563	98.3	1.9
GB	1.034	1.001	1.056	1.028	1.006	1.082	100.0	3.3

Table 3
Intra- and inter-day precisions

Sample	Intra-day		Inter-day	
	Mean (μg/vial)[a]	RSD (%)	Mean (μg/vial)[b]	RSD (%)
BB	528.8	2.7	519.9	2.5
GC	413.9	3.8	408.0	2.5
GA	716.3	2.0	706.1	1.6
GB	408.1	2.0	399.4	1.1

[a] Mean, $n = 6$.
[b] Mean, $n = 4$.

Table 4
Quantitation of the sample

Lot		Recovered amounts (μg/vial)					Mean (μg/vial)	RSD (%)
990401	BB	598.3	603.0	583.6	599.6	592.0	595.3	1.3
	GC	439.3	458.0	454.6	444.6	440.9	447.4	1.9
	GA	823.6	857.2	836.0	827.6	834.6	835.8	1.6
	GB	475.6	525.1	474.8	480.0	496.3	490.4	4.3
990402	BB	598.6	601.6	601.7	602.2	596.5	600.1	0.4
	GC	452.1	457.1	479.2	449.6	482.4	464.1	3.3
	GA	870.0	887.8	923.8	886.4	911.6	895.9	2.4
	GB	541.4	559.8	531.9	559.1	519.4	542.3	3.2
990403	BB	598.9	603.0	589.3	570.3	588.8	590.0	2.1
	GC	496.6	514.0	472.3	464.0	462.6	481.9	4.7
	GA	820.6	833.4	851.2	827.9	846.6	836.0	1.5
	GB	464.2	489.5	499.6	509.0	478.8	488.2	3.2

and detecting BB, GC, GA, and GB in GBE injections. According to the above purification procedure, five 10 ml samples of GBE injections were extracted and purified and the amounts in Table 4 were determined with the calibration curves shown in Table 1.

4. Conclusions

This sample purification procedure offered in the proposed method consisted of the liquid extraction and one cleanup step, and the performance of ELSD is particularly appropriate for the analysis of terpene compounds with low UV absorption. Good precision and accuracy were achieved. This simple, low-cost, and reliable assay is suitable for the routine quantitation of BB, GC, GA, and GB in GBE injections.

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