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Short communication

Optimization of extraction technique and validation of developed RP-HPLC-ELSD method for determination of terpene trilactones in *Ginkgo biloba* leaves

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ABSTRACT

Terpene trilactones are potent and selective antagonists of platelet activating factor. The present study deals with standardization of efficient extraction method and validation of newly developed simple, sensitive and rapid reversed phase high performance liquid chromatographic method with evaporative light scattering detection (RP-HPLC-ELSD) method for the quantitative determination of ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), ginkgolide J (GJ) and bilobalide (BB) within 8 min in *Ginkgo biloba* leaf extract. The analysis was conducted on a Zorbax RP-C_{18} column with gradient elution of methanol–water–tetrahydrofuran. The method was validated for accuracy, precession, limit of detection and quantification. The drift tube temperature of evaporative light scattering detector was set to 90 °C and nitrogen flow rate was 1.5 standard liter/min (SLM).

1. Introduction

The use of botanical dietary supplements has experienced a steady growth recently. Extract of leaves of *Ginkgo biloba* is among the top 10 commercial products in the world [1]. The *Ginkgo* tree has been of interest to mankind for more than 2000 years, making it one of the oldest known medicinal plants. Pharmacological properties of its leaves include radical scavenging, improved blood flow, vasoprotection and anti-platelet activating factor activity [2]. *Ginkgo* extract is also known for its anti-ischemic [3–5], antioxidant [6–8] and anticonvulsant [9] properties. Moreover, the increasing research evidences support that the bioactive components of *Ginkgo* extract have significant therapeutic effects on age related physical and mental deterioration and on cerebral vascular insufficiency, e.g., Alzheimer's and cardiovascular disease [10–12]. Among its constituents, bilobalide (BB), ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC) and ginkgolide J (GJ) (Fig. 1) along with flavonoids have been identified as the active constituents [13]. Ginkgolides (diterpene) and bilobalide (sesquiterpene) collectively known as terpene trilactones (TTLs) are not only structurally unique with highly oxygenated functions but also main bioactive constituents of *G. biloba* which imparts broad spectrum of pharmacological activities to the plant.

Due to the above reasons, the terpene trilactones enriched extract is the valuable source of herbal products. Therefore, the standardized plant extract is important for commercial production. Developing a fast and efficient way for production of high quality botanical product has become an issue of concern in pharmaceuticals and food industries. For extract preparation, selection of appropriate extraction method is of key consideration. Presently number of reports are available on the extraction of terpene trilactones from *G. biloba* leaves using resins, supercritical fluid extraction, liquid–liquid extraction, pressurized water extraction, nanofilteration etc. [13]. These methods are either tedious, require large amount of solvents, use of costly resins and equipments or have poor recoveries. Few extraction techniques like ultrasonic assisted extraction (UAE), pressurized solvent extraction (PSE), microwave assisted extraction (MAE) etc. with low solvent consumption in less extraction period have been developed during the last three decades and can be adopted for routine analysis. However, comparative studies of these methods, especially for the extraction of terpene trilactones from *G. biloba* have not been attempted previously.

Analytical methods for the analysis of TTLs in *G. biloba* by gas chromatography (GC) with mass spectrometry (MS) and flame ionization detector (FID), nuclear magnetic resonance (NMR), liquid chromatography with mass spectrometry (LC–MS) and reverse phase high performance liquid chromatography (RP-HPLC) have been reported in the literature [14–26]. GC, NMR and LC–MS have proved to be valuable detection methods due to their sensitivity and tandem mass spectrometry (MS–MS) facilitates exclusive analysis of selected peaks of interest with unequivocal peak identification. These analytical methods require complex sample preparation, highly trained personnel and maintenance make these detec-

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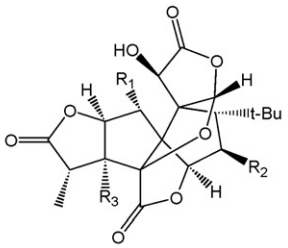

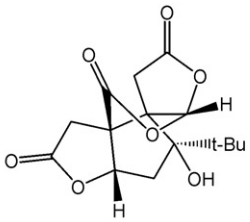

Bilobalide (BB)

	R_1	R_2	R_3
Ginkgolide A (GA)	H	H	OH
Ginkgolide B (GB)	OH	H	H
Ginkgolide C (GC)	OH	OH	OH
Ginkgolide J (GJ)	H	OH	OH

Fig. 1. Structure of terpene trilactones of *G. biloba*.

tion methods currently impractical for routine analysis [16,27]. HPLC is the simplest tool for the quantitative determination of the bioactive constituents in pharmaceutical industry. As TTLs are poor chromophores, it makes UV detection difficult, especially when analyzing crude *Ginkgo* extracts. Extensive clean-up procedures are usually required prior to the analysis of leaf extracts and formulations [27–29]. Refractive index (R.I.) detection has been used successfully but unstable baseline and low sensitivity [19,29,30] remain the problem. On the other hand, the evaporative light scattering detection (ELSD) system has gained popularity for the analysis of non-volatile compounds that possess poor chromophores. A number of reports have appeared for the determination of TTLs in different *Ginkgo* products using HPLC with ELSD detection [29,31–35]. Dubber and Kanfer have analyzed the *G. biloba* solid oral dosage using ELSD with separation time of 14 min. The authors have also reported validation of the method but the sample requires clean-up prior to analysis [35].

In present study, different extraction methods were compared and optimized for the high extraction yield of TTLs. A validated system permitting the separation of terpene trilactones within 8 min without the need of sample clean-up prior to analysis from the crude extract of *G. biloba* leaves has been developed.

2. Experimental

2.1. Materials

The leaves of *G. biloba* were collected from six different regions having varying altitude (236–2062 m) of Punjab, Uttrakhand, Assam and Himachal Pradesh in India during July–August 2006. The samples were authenticated by biodiversity department of Institute of Himalayan Bioresourse Technology, Palampur (Himachal Pradesh), India and stored at 25 °C until further use. GJ (98.20%), GC (99.60%), BB (99.90%), GA (99.90%) and GB (89.50%) were purchased from Chromadex, Life Technology, India. The peak purity of the reference compounds was checked by HPLC, NMR and MS. All HPLC grade solvents; methanol, water and tetrahydrofuran (THF) were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Standard solutions

A stock solution of ginkgolides and bilobalide of 0.5 mg/mL was prepared in methanol. Stock solution containing five analytes was diluted to get final concentrations in the range of 500–8000 μg/mL for establishing calibration curves. Injection volume used for each run was 20 μL. All stock solutions and work solutions were stored in a refrigerator (4 °C) until further use.

2.3. Comparison of extraction techniques

Comparison of six extraction techniques, i.e., UAE, percolation (PERC), orbital shaking (ORSH), refluxing (REFLUX), soxhlet (SOXH) and accelerated solvent extraction (ASE) were carried out using single optimized extraction solvent mixture with the same solid to solvent ratio. All the extracts were analyzed by HPLC for the extracted content of individual terpene trilactone.

2.3.1. Ultrasound-assisted extraction

Optimization of solvent for extraction of TTLs was carried out using UAE as it was proved to be the quicker and time saving technique. Different solvent systems had been used for the extraction, e.g., methanol:water, acetone:water and ethanol:water. Of these systems, mixture of methanol and water was reported to be best for the extraction of terpene trilactones [27]. In the present study five different solvent systems, i.e., pure methanol, 90% aq. methanol, 70% aq. methanol and 50% aq. methanol were used for the extraction using UAE. Dried leaves (2 g) with 20 mL of solvent were extracted in ultrasonic bath at 40 °C for 15, 30, 45, 60 , and 90 min. The extracts were filtered, dried and prepared in 2 mL of HPLC grade methanol for analysis and injected in HPLC after filtration through 0.45 μm membrane.

2.3.2. Cold and hot extraction

Test powder (2 g) was packed in a percolator and the solvent (20 mL of 90% aq. methanol) percolated through the powder packing and collected. The collected percolations were filtered and residues were washed with extracting solvent. Combined filtrates were dried in rotary-evaporator under vacuum. Dried extract was prepared for chromatographic analysis.

In orbital shaker, 2 g plant material immersed in 20 mL of 90% methanol was extracted at 160 rpm at 28 °C for overnight. For refluxing and soxhlet extraction techniques, plant material (2 g) was extracted at 60 °C for 6 h with the optimized solvent. All the extracts obtained were filtered, dried and prepared for analysis.

2.3.3. Pressurized solvent extraction

ASE is a registered technique of PSE that combine elevated temperature and pressures with solvents to achieve efficient extraction. Powdered plant material (2 g) was placed in the extraction cell of ASE (ASE 200, Dionex Corporation, Sunnyvale, CA). As pressure and temperature play significant role in the extraction of constituents from the plant matrix therefore, only these were optimized for the analysis. The extraction was conducted under following conditions: solvent: 90% methanol; temperature: 40, 50, 60 and 70 °C; static time: 2 min; purge time: 60 s; static cycles: 2; pressure: 600, 1200 and 2400 psi.

2.4. HPLC instrumentation and chromatographic conditions

HPLC analysis was performed on a Shimadzu Prominence system equipped with LC-20AT quaternary gradient pump, PL-ELS 1000 evaporative light scattering detector from Polymer Laboratories (Church Stretton, UK), CBM-20A communication bus module, CTO-10AS vp column oven, SIL-20AC auto sampler and Shimadzu LC solution software (ver. 1.21 SP1). Separation was achieved on Zorbax Extend C-18 column (250 mm × 4.6 mm, 5 μm) from Agilent (USA). The temperature of the column was set at 35 °C. The mobile phase was consisted of 13% aq. THF (A)–methanol (B) in gradient elution with flow rate of 1 mL/min. The gradient elution was programmed as follows: 0–10 min, 33% B; 10–20 min, 33–80% B; 20–25 min, 80–33% B; 25–30 min, 33% B. The detection of analytes was carried out by using evaporative light scattering detection technique which detects the organic molecules by its mass and hence useful in quantitative determination of non-UV sensitive

Table 1
Content variation of ginkgolides and bilobalide in different extraction techniques.

Compound	ASE 1200 (%, w/w)	SOXH (%, w/w)	ORSH (%, w/w)	PERC (%, w/w)	UAE (%, w/w)	REFLUX (%, w/w)
GJ	0.02	0.02	0.01	0.02	0.03	0.02
GC	0.14	0.11	0.08	0.16	0.21	0.26
BB	0.65	0.47	0.39	0.78	1.02	1.14
GA	0.11	0.09	0.07	0.13	0.17	0.19
GB	0.09	0.07	0.04	0.10	0.15	0.14
Total	1.01	0.75	0.59	1.19	1.58	1.74

ASE 1200, accelerated solvent extraction at 1200 psi; SOXH, soxhlet; ORSH, orbital shaker; PERC, percolation; UAE, ultrasonic assisted extraction; REFLUX, reflux.

compounds. The drift tube and evaporator temperatures for ELSD were set at 90 and 110 °C, respectively and nebulizing gas (N_2) flow rate was 1.5 standard liter/min (SLM).

3. Results and discussion

3.1. Optimization of extraction technique

In order to obtain quantitative yield by ultrasonic system the extraction solvent was optimized by examining different solvent systems such as aq. methanol (50%, 70% and 90%) and 100% methanol. The yield of analytes obtained from 90% and 50% aq. methanol was comparable but 90% aq. methanol produced cleaner chromatogram. Low yields were recorded with 70% aq. methanol and methanol in extraction. Extraction time was also investigated for optimum yield. With optimized conditions all the analytes were extracted within 15 min, longer period of ultrasonic irradiation decreased the analyte yield.

Other extraction techniques like soxhlet, percolation, refluxing, orbital shaking, modern ASE were applied and compared to bring out the best extraction procedure for terpene trilactones with the optimized solvent. In ASE, maximum yield of TTLs was obtained at 1200 psi and 50 °C. Refluxing and ultrasonication were found to show comparable results, but refluxing is more time consuming procedure, therefore, ultrasonication was considered as the best method for the extraction of the ginkgolides and bilobalide. The concentrations obtained using different extraction methods of all the analytes is shown in Table 1.

3.2. Optimization of chromatographic conditions

For optimization of chromatographic conditions, better resolution was recorded with methanol–water–THF system for all active components than methanol–water or acetonitrile–water system. The peak resolution was also recorded with variation in the column temperature. Column temperature was optimized systematically from 25 to 40 °C, and it was observed that all the components achieved a baseline separation at 35 °C. As a result, methanol–water–THF was optimized as the eluting solvent system, and column temperature at 35 °C. Optimized conditions were also applied on different RP-C_{18} columns like Zorbax (Agilent), LiChrosphere (Merck) and Phenomenex (LUNA C_{18} Torrance, CA) having same dimensions (250 mm × 4.6 mm × 5 μm) but, the best resolution was obtained with Zorbax column. Operating conditions for ELSD were optimized according to the data computed with the ELSD software: the nebulizing gas flow rate set at 1.5 SLM, The drift tube and evaporator temperatures for ELSD were set at 90 °C and 110 °C respectively. All HPLC and ELSD parameters like optimal separation, high sensitivity and good peak shape of the analytes were standardized. Four ginkgolides and bilobalide, viz. GJ (RT: 4.46 min), GC (RT: 4.99 min), BB (RT: 5.71 min), GA (RT: 6.40 min) and GB (RT: 7.57 min) were well resolved. The representative chromatograms of the standard mixture and sample of *G. biloba* are shown in Figs. 2 and 3. The chromatographic peaks were identified by comparing their retention times with reference compounds and spiking of samples with

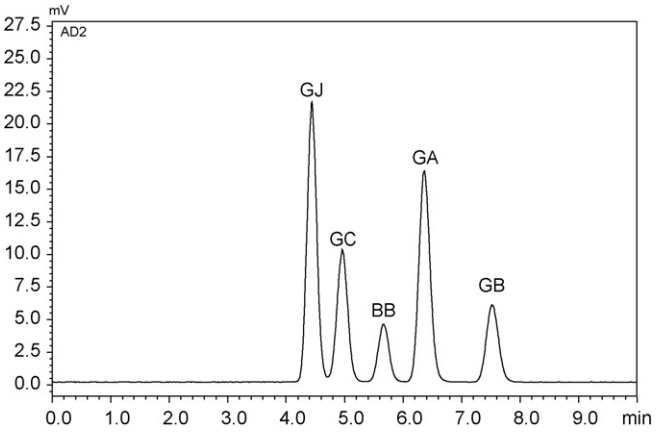

Fig. 2. HPLC-ELSD chromatogram of standard mixture of TTLs.

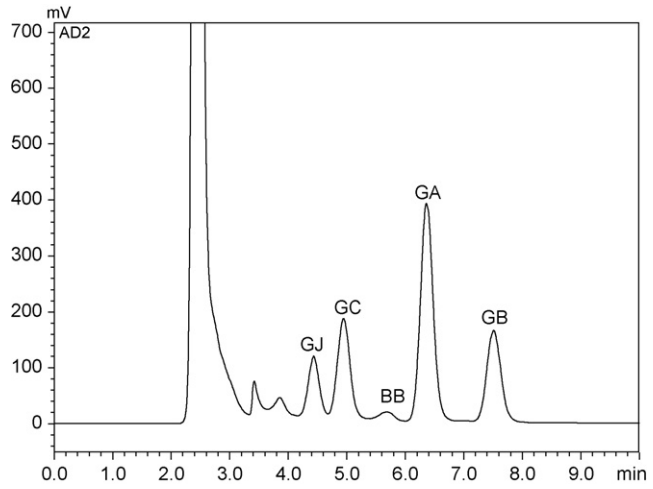

Fig. 3. HPLC-ELSD chromatogram of *G. biloba* sample collected from Dehradoon.

the reference compounds. The results indicate that ginkgolides and bilobalide were well resolved and their quantitative determination in *G. biloba* was possible.

3.3. Analytical method validation

The developed high performance liquid chromatographic method with evaporative light scattering detection (HPLC-ELSD) method for quantitative evaluation of ginkgolides and bilobalide was validated according to International Conference on Harmonization (ICH) guidelines [36].

3.3.1. Precision and accuracy

The precision and accuracy of the method were determined by spiking the sample collected from Dehradoon, with accurate quantity of standards. The mean recovery was calculated on three

Table 2
Inter-, intra-day precision and recovery (%).

TTLs	Intra-day precision ($n = 3$), %RSD						Inter-day precision ($n = 6$) %RSD		Recovery (%)	
	Day 1		Day 2		Day 3					
	RT*	PA**	RT*	PA**	RT*	PA**	RT*	PA**	Spiked amount (μg)	% Recovered
GJ	0.07	3.21	0.09	3.01	0.40	2.21	1.80	2.95	2.50	96.56
									5.00	98.29
									7.50	93.53
GC	0.14	3.25	0.05	2.01	0.47	3.39	1.97	3.60	2.50	96.31
									5.00	93.67
									7.50	94.43
BB	0.11	2.39	0.09	2.50	0.48	2.41	1.50	3.12	2.50	95.15
									5.00	95.45
									7.50	98.74
GA	0.18	2.55	0.14	3.41	0.48	2.85	3.18	3.14	2.50	94.60
									5.00	93.72
									7.50	97.27
GB	0.06	3.32	0.12	3.62	0.46	2.98	3.34	2.94	2.50	97.80
									5.00	96.90
									7.50	99.48

* RT, %RSD of retention time.
** PA, %RSD of peak area.

Table 3
Regression equation, limit of detection and limit of quantification of TTLs.

Analytes	Regression equation	Linearity range (μg/mL)	r^2	LOD (μg/mL)	LOQ (μg/mL)
GJ	$57572x - 68075$	500–8000	0.9961	250.0	825.0
GC	$24879x - 17986$	500–8000	0.9942	300.0	990.0
BB	$8916.7x - 3728.8$	500–8000	0.9898	350.0	1155.0
GA	$42877x - 38608$	500–8000	0.9972	250.0	825.0
GB	$18052x - 17627$	500–8000	0.9968	400.0	1320.0

assays for the standards. The developed analytical method showed good accuracy with the satisfactory recovery in the range of 93.53–99.48% (Table 2). Measurement of intra- and inter-day variability was utilized to determine the precision of newly developed method. The intra-day variation was determined by analyzing in duplicate the mixed standard solution for five times within 1 day. While, for inter-day variability test, the solution was examined in duplicate for alternate 3 days. The percentage relative standard deviation (%RSD) was taken as a measure of precision. The precision results showed the low values (less than 5.0%) of intra- and inter-day %RSD of retention time and peak areas as shown in Table 2.

3.3.2. Calibration curves, linearity and selectivity

Linear calibration curves were drawn using five concentration points on each of the 3 days and plotting the peak areas versus the concentration of each analyte. The range and correlation coefficients are presented in Table 3. All calibration curves showed good linear regression in the range $r^2 = 0.9898–0.9972$. The selectivity of the method was determined by analysis of standard compounds and samples.

3.3.3. Limit of detection and quantification

Stock solution containing reference compounds was diluted to give series of appropriate concentrations with methanol and the aliquots of the diluted solutions were injected. The limits of detection (LOD) and limit of quantification (LOQ) for each detected analyte were calculated with corresponding standard solution on the basis of signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD's and LOQ's for terpene trilactones were found to be in the range of 250.0–400.0 μg/mL and 825.0–1320 μg/mL, respectively (Table 3).

3.4. Quantification of terpene trilactones in Ginkgo samples

The developed HPLC method was applied to analyze six samples collected from different locations in India. In all the samples BB was recorded to be in higher amount (0.19–0.38%) whereas GA was found to be maximum (0.59%) in Dehradoon sample. Total content of terpene trilactones showed significant variation (0.19–1.89%) in the samples collected from different altitudes and was observed to be maximum in Dehradoon sample (1.89%). It was also observed that from some of the locations ginkgolide J (Nanital, Darjeeling

Table 4
Contents of GJ, GC, BB, GA and GB in *G. biloba* from different locations in India.

Altitude (in m asml*)/location	GJ (%, w/w)	GC (%, w/w)	BB (%, w/w)	GA (%, w/w)	GB (%, w/w)	Total content (%, w/w)
236 Patiala (Punjab)	0.02	0.07	0.19	0.02	0.11	0.42
637 Dehradoon (Uttrakhand)	0.23	0.50	0.13	0.59	0.44	1.89
1705 Nanital (Uttrakhand)	nq**	0.05	0.38	0.01	0.04	0.46
2038 Darjeeling (Assam)	nq**	nq**	0.19	nq**	nq**	0.19
2040 Raniket (Uttrakhand)	nq**	nq**	0.20	nq**	0.01	0.21
2062 Shimla (Himachal Pradesh)	0.01	0.03	0.21	nq**	0.01	0.26

* In meters above mean sea level.
** Not quantified.

and Raniket), ginkgolide C (Darjeeling and Raniket), ginkgolide A (Darjeeling, Raniket and Shimla) and ginkgolide B (Darjeeling) were not in detectable range. On the basis of above results (Table 4) it can be mentioned that the *G. biloba* plant growing in middle altitude from 600 to 1710 m can provide good quality products for the pharmaceutical industries.

4. Conclusion

Terpene trilactones are the unique cage like structural compounds showing broad spectrum of pharmacological activities. Therefore, the present extraction and HPLC-ELSD method has been applied for the determination of terpene trilactones from different locations in India. The results of the analysis, undertaken according to the ICH guidelines, revealed that method is selective. The proposed method is simple, accurate, precise, specific and has ability to separate TTLs from *G. biloba* in a short time. The developed HPLC-ELSD method has been satisfactorily verified for its accuracy and selectivity and can be used for quality control of herbal formulations. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments, such as LC–MS or GC–MS.

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