Analysis of Terpene Lactones in a Ginkgo Leaf Extract by High-Performance Liquid Chromatography Using Charged Aerosol Detection

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Received November 2, 2009; Accepted December 17, 2009; Online Publication, March 7, 2010
[doi:10.1271/bbb.90802]

A new HPLC method using charged aerosol detection was developed for the determination of terpene lactones in a Ginkgo leaf extract. The linearity of the standard curves was excellent (r > 0.999). The repeatability of the method was less than 3%, and its reproducibility was less than 5% for each analyte. The limit of detection was between 0.087 and 0.45 μg/ml. The developed method was applied to the analysis of terpene lactones in Ginkgo leaf products distributed in the Japanese market. The results suggest that some health food products contained approximately equivalent amounts of terpene lactones to those in the medical product and that the proportion of terpene lactones varied in each health product.

Key words: Ginkgo biloba; ginkgolide; bilobalide; HPLC; charged aerosol detection

Ginkgo biloba is one of the oldest living tree species. The name “Ginkgo” is derived from a Japanese mispronunciation of the Chinese name for the plant “yin-kuo” (silver apricot).1) Ginkgo leaves have been used in China for about 5000 years to treat such lung ailments as asthma and bronchitis and as a remedy for cardiovascular diseases. The pharmacological effects of a Ginkgo leaf extract on the circulatory system were first reported in the western world in 1966. The extract is presently used as a herbal medicine for treating dementia and other diseases in many countries throughout the world.1–4)

It is known that terpene lactones (Fig. 1) are the main active components in a Ginkgo leaf extract, as well as flavonoids. Some reports have shown that terpene lactones improved the blood circulation by its antiplatelet activity, increased the activities of antioxidative enzymes and improved cell viability.5) Ginkgolides A and B have also decreased corticosteroid synthesis, and subsequently, the circulating levels of glucocorticoids. The pharmaceutical effects of a Ginkgo leaf extract are considered to result from the various activities of these compounds.2,4)

Since the faint UV absorption of terpene lactones hampers UV-dependent detection, various alternative methods have been reported for analyzing these compounds.3) HPLC-Oriented assays of the terpene lactones in a Ginkgo leaf extract have been established by refractive index detection (RID), a simple and inexpensive detector, in German Pharmacopoeia.6) The HPLC/MS method with outstanding sensitivity and selectivity has been reported for the quantitative determination of Ginkgo terpene lactones.7–11) Evaporative light scattering detection (ELSD), in American Herbal Pharmacopoeia1) and previous reports,12–16) is also highly sensitive when used in conjunction with efficient gradient elution. HPLC/RID, however, often suffers from either its low sensitivity or compatibility issue with gradient elution. Furthermore, HPLC/MS is too expensive for routine use and requires highly trained people for operation and maintenance, and HPLC/ELSD sometimes offers insufficient sensitivity.

We present here a novel analytical HPLC method for terpene lactones in a Ginkgo leaf extract using charged aerosol detection (CAD), which was first introduced by Dixon and Peterson,17) and has been used for the analysis of carbohydrates, lipids, amino acids and polymers which have little UV absorption.18) The CAD method has better sensitivity and repeatability compared with RID or ELSD, and is suitable for gradient elution. Furthermore, CAD can be handled more easily than MS method because of fewer parameters and simpler equipment. We also applied this analytical method for evaluating the quality of health foods containing a Ginkgo leaf extract in the Japanese market.

Materials and Methods

Materials. Standards for bilobalide and ginkgolides A, B, and C were obtained from Nagara Science (Gifu, Japan) and that for ginkgolide J from ChromaDex (Santa Ana, CA, USA). Ethyl acetate and methanol (both HPLC/MS grade) and KH2PO4 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were obtained from Kanto Chemical (Tokyo, Japan). Purified water was generated by using a Milli-Q deionization unit from Millipore (Billerica, MA, USA). The stock solutions of terpene lactones were prepared in methanol and serially diluted to the desired concentration with 50% v/v methanol as treatment if necessary.
the diluent. A 20 ml Chem Elut cartridge was obtained from Varian (Walnut Creek, CA, USA). Sixteen commercial Ginkgo leaf products distributed as health foods were purchased from the Japanese market (samples A–P), and a medical Ginkgo leaf extract (sample Q) was obtained from Dr. Willmar Schwabe Pharmaceuticals through a German pharmacy.

**Instrumentation.** The terpene lactones were analyzed by a Prominence HPLC system consisting of a vacuum degasser, binary pump, autosampler and column heater from Shimadzu (Kyoto, Japan), and a Corona CAD system from ESA Analytical (Aylesbury, Buckinghamshire, UK). The CAD range was set to 100 pA and the nitrogen inlet pressure was set to 0.24 MPa in accordance with the operating manual. LC Solution software (Shimadzu) was used to control the instruments and process data.

**Sample preparation.** Samples for the HPLC/CAD analysis were prepared on the basis of the German Pharmacopoeia with a few modifications. HPLC samples of the commercial Ginkgo leaf products were each prepared by mixing the pulverized contents of 20 tablets or the internal contents of 20 capsules. The powder (80 mg) was placed in a 50-ml beaker, and dissolved in 15 ml of a 0.67 mmol/l phosphate buffer solution (pH 5.8) while stirring. The solution was transferred to a Chem Elut cartridge. After rinsing the beaker with 5-ml portions of phosphate buffer solution, the solution was also transferred to the Chem Elut cartridge. The mixture was allowed to stand for 15 min, before eluting with 100 ml of ethyl acetate. The eluate was evaporated, and the solvent residue was completely removed by using nitrogen gas. The final residue was dissolved in 2.5 ml of 30% v/v methanol, and filtered through a 0.45-μm PVDF membrane filter. The resulting

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**Fig. 1.** Structures of the Major Terpene Lactones in *Ginkgo biloba.*

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgolide A</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Ginkgolide B</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Ginkgolide C</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Ginkgolide J</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>

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**Analysis of Bioactive Components in a Ginkgo Leaf Extract**

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**Fig. 2.** HPLC/CAD Chromatograms for Sample A Containing a Ginkgo Leaf Extract.

Peak identification: 1, bilobalide; 2, ginkgolide J; 3, ginkgolide C; 4, ginkgolide A; 5, ginkgolide B. Mobile phase conditions: a, methanol/water, 50/50; b, methanol/water, 40/60; c, methanol/water, 30/70; d, methanol/water, 30/70; 40 μg/ml of bilobalide, 20 μg/ml of ginkgolide A and C, 10 μg/ml of ginkgolide B, and 5 μg/ml of ginkgolide J standard solutions.
solution was diluted with 30% v/v methanol and transferred to a silane-untreated vial to maintain stability for the HPLC/CAD analysis.

Chromatographic conditions. All experiments used a 150 mm × 4.6 mm Mightysil RP-18 column (Kanto Chemical) packed with 5-μm particles after conditioning with 30% v/v methanol. The mobile phase consisted of water as eluent A and methanol as eluent B. The flow rate was set to 1.0 ml/min and the column temperature was controlled at 40 °C. An autosampler was used for injection, with the injection volume set at 10 μl. The mobile phase condition with the following gradient elution was used in the final optimized method: 30% B from 0 to 20 min, then increased to 70% B in 5 min, held at 70% B from 25 to 30 min, followed by a decrease to 30% B in 5 min, and a re-equilibration from 35 to 40 min at 30% B.

Table 1. Linearity (r), Repeatability (RSDa%), Reproducibility (RSDb%), LOD, and LOQ for the Analysis of Terpene Lactones by HPLC/CAD

<table>
<thead>
<tr>
<th>Compound</th>
<th>r (log–log)</th>
<th>RSDa% (n = 6)</th>
<th>RSDb% (n = 3 for 3 d)</th>
<th>LOD (μg/ml)</th>
<th>LOQ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilobalide</td>
<td>&gt; 0.999</td>
<td>1.4</td>
<td>4.8</td>
<td>0.087</td>
<td>0.29</td>
</tr>
<tr>
<td>Ginkgolide A</td>
<td>&gt; 0.999</td>
<td>1.5</td>
<td>2.2</td>
<td>0.23</td>
<td>0.77</td>
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<tr>
<td>Ginkgolide B</td>
<td>&gt; 0.999</td>
<td>1.5</td>
<td>4.5</td>
<td>0.45</td>
<td>1.5</td>
</tr>
<tr>
<td>Ginkgolide C</td>
<td>&gt; 0.999</td>
<td>2.7</td>
<td>3.8</td>
<td>0.13</td>
<td>0.43</td>
</tr>
<tr>
<td>Ginkgolide J</td>
<td>&gt; 0.999</td>
<td>1.9</td>
<td>1.3</td>
<td>0.20</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Fig. 3. Log–Log Transformed Response Plots for the Terpene Lactones.

Fig. 4. HPLC/CAD Chromatograms with Gradient Elution for the Terpene Lactones Found in Ginkgo Leaf Products. Samples: A, C, H, L, P, Q from top to bottom. Peak identification: 1, bilobalide; 2, ginkgolide J; 3, ginkgolide C; 4, ginkgolide A; 5, ginkgolide B.
Results and Discussion

**HPLC separation**

In order to find simple and separable mobile phase conditions for the analysis of five major terpene lactones (bilobalide and ginkgolides A, B, C, and J), the methanol/water ratio was examined with health food A as the test sample. The result gave good separation of the five terpene lactones with a methanol/water ratio of 30/70 (Fig. 2). Gradient elution was adopted after the elution of these terpene lactones to shorten the whole HPLC run-time. Since it is well known that the CAD response depends on the organic content of the mobile phase, the composition was kept stable during the elution of the five terpene lactones. The gradient elution as described enabled the whole HPLC run-time to be shortened to 40 min, whereas over 90 min was required for completing a single analysis with isocratic elution.

**Linearity**

Response plots of the analyte concentration versus area response of CAD show a nonlinear response in agreement with theory. After log–log transformation, as described in the previous report, linear response curves were obtained with a high correlation coefficient ($r > 0.999$). The ranges of concentration were 8–40 µg/ml for bilobalide, 4–20 µg/ml for ginkgolides A and C, 2–10 µg/ml for ginkgolide B, and 1–5 µg/ml for ginkgolide J (Table 1, Fig. 3).

**Precision**

Sample A was used for the analyses in order to evaluate the repeatability and reproducibility of the method. Repeatability was evaluated by performing six analyses on the same day under the same conditions. Reproducibility was evaluated by performing three analyses on three different days. The results are summarized in Table 1. The method achieved high precision, with RSD 1.4–2.7% for repeatability and RSD 1.3–4.8% for reproducibility.

**Sensitivity**

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by using the standard solutions. LOD is defined as the concentration leading to signal/noise = 3, and LOQ as that leading to signal/noise = 10. The LOD values were in the range of 0.087–0.2 µg/ml and LOQ values in the range of 0.29–1.5 µg/ml (Table 1). The high sensitivity of this method enabled us to determine even the minor component, ginkgolide J, with high precision.

**Analysis of commercial Ginkgo leaf extracts**

The HPLC/CAD-based method, which provided sufficient precision and sensitivity to analyze Ginkgo leaf extracts, was applied to analyze the terpene lactones in products containing Ginkgo leaf extracts. Typical chromatograms are shown in Fig. 4. The concentration of five Ginkgo terpene lactones in sixteen commercial products was estimated from the standard curves presented in Fig. 3. Taking into account the suggested usage on the label, the amount of each compound was expressed as the content for maximum daily intake (Fig. 5). The amount of medical sample Q is within the range of medical standards in the German Pharmacopoeia. Such health food products as samples A, E, F, J, and K included amounts of total terpene lactones nearly equal to the medical standards. On the other hand, samples B and P contained an extremely low level of terpene lactones. We also observed that the proportion of terpene lactones varied in each health product and that the terpene lactone proportion in some health products was quite different from that in the medical product. Since a Ginkgo leaf extract is a natural product, the difference is considered to have been dependent upon the climate, soil, time of harvest, and other agricultural/manufacturing conditions.

Acknowledgments

This work was supported by a grant for research on publicly essential drugs and medical devices from The Japan Health Sciences Foundation.

References