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Analysis of Ginseng Saponins by HPLC with Photoreduction Fluorescence Detection

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Abstract □ A new high performance liquid chromatographic procedure is described for the analysis of ginseng saponins. Ginseng saponins were separated on Lichrosorb NH_2 column and anthraquinone-2,6-disulfonate (AQDS) solution was added to the column effuent. The effluent was passed through 1.5 m-PTFE capillary coiled around 10 W-UV lamp to reduce AQDS to highly fluorescent 9,10-dihydroxyanthracene-2,6-disulfonate which was detected by fluorescence detector. The detection limit for the ginsenoside Rg_1 by this method was found to be about 350 ng, the dynamic linear range was 10^2 and the correlation coefficient of the calibration curve was 0.9999.

Keywords □ Photoreaction HPLC, anthraquinone-2,6-disulfonate, photoreduction fluorescence, analysis of ginsenoside, *Panax ginseng*.

Panax ginseng C.A. Meyer (Araliaceae) has long been used as a traditional tonic medicine in the Orient. The HPLC and GLC methods are commonly used for the quantitation of ginseng saponins, the main constituents of ginseng[1]. Although HPLC is a good method for the analysis of individual ginsenoside, it is necessary to develop a proper detection method which has high sensitivity and selectivity. Since ginseng saponin is a poor chromophore, short UV wavelength, typically 203 nm, has to be adapted to detect it. However, at this wavelength, many compounds which absorb UV radiation interfere with ginsenosides and the highly pure solvent is needed. The refractive index (RI) detector lacks sensitivity and it is hard to set proper operating conditions.

Recently, post-column photochemical reaction has been successfully applied to LC detection, since this method has demonstrated the selectivity and sensitivity needed for the modern analysis. Photochemical reaction has many advantages to the normal thermal post-column reaction[2-4]. Since photochemical reaction proceeds *via* the free radical intermediate, the reaction rate is very high. Photoreactioin requires photon instead of chemical reagent, which makes the reaction device more simple and minimizes band broadening. Furthermore, this method has a good selectivity to the analyte that is able to undergo photoreaction.

In photoreduction detection, photoreductive compound usually is added to HPLC eluent. AQDS was used in this experiment, of which photoreduction process is illustrated in Fig. 1[4-6]. AQDS which was excited to the singlet state (S_1) by UV irradiation converts to the triplet state (T_1) through a rapid intersystem crossing. The triplet state is biradical in character and undergoes reactions similar to those of alkoxy radical. The AQDS in the triplet state reacts with proton-donating substrates (e.g. alcohol, ether, aldehyde, saccharide etc) to yield dihydroxyanthracene derivative. As a result, AQDS which is very weakly fluorescent (quantum yield : *ca.* 10^{-5}) turns to highly fluorescent 10-dihydroxyanthracene-2,6-disulfonate (quantum yield : *ca.* 0.7) by

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the irradiation of UV light in the presence of proton-donating substrates[7]. This reaction is dependent on the concentration of substrate, therefore it is possible to determine the amount of substrate through the measurement of fluorescence intensity of 9,10-dihydroxyanthracene-2,6-disulfonate. This method was developed by Birks and Gandelman who applied it to the analysis of saccharides and cardiac glycosides[4,5]. In this paper, we report the application of this method to the analysis of ginseng saponins.

EXPERIMENTAL METHODS

Materials

Anthraquinone-2,6-disulfonic acid, disodium salt (FW 412.31, mp. 325℃) was purchased from Aldrich and was recrystallized from water and washed with acetonitrile. Th stock solution which contains 2.5g/*l* of aqueous AQDS was stored in a brown glass bottle. Distilled and deionized water and HPLC grade acetonitrile were used for the experiment. Ginseng was purchased from the local herbal drug market in Seoul.

Chromatographic apparatus

The instruments empolyed were Shimadzu HPLC pump (LC-9A) equipped with 20 μ*l* loop injector (model 7125, Rheodyne, USA) connected to a photochemical reactor. Shimadzu fluorescence detector (RF-535) was empolyed (excitation at 400 nm; emission at 525 nm). The chromatographic data was processed by Shimadzu C-R4A Chromapac integrator. Lichrosorb NH_2 column (250 mm×4 mm i.d., 5 μm particle diameter, Merck) was used at room temperature. Hitachi L-4200 UV detector was empolyed to compare the detection methods.

Photochemical reactor

10 W-UV lamp (2.5 cm×32 cm cylinder type, Sam-Gong Co. Korea), which is usually used in UV sterilizer for tableware, was purchased at the local electronics market. The 1.5 m PTFE capillary tube (0.32 mm i.d.×2.0 mm o.d.) was coiled around the lamp[8], and the reactor was wrapped with aluminum foil to increase the photon flux of lamp by reflection.

Chromatographic conditions

To optimize the reaction condition, Lichrosorb RP-18 column (Merck) was used as a stationary phase and AQDS was directly added to the eluent. Photoreaction time was changed by varying the flow rate of the eluent. For the analysis of ginseng saponins, Lichrosorb NH_2 column, and aqueous acetonitrile solution (acetonitrile/water=80/20, 0.7 m*l*/min) were used. Aqueous AQDS solution (1.0×10^{-3} M, 0.3 m*l*/min) was mixed to the column effluent using three-way mixing tee, and the mixture was passed through 1..5 m-PTFE tube coiled around 10 W-UV lamp. The mobile phase was purged with N_2 to remove oxygen in the eluent[5].

Photoreaction condition

To search for the optimal photoreaction condition, 5 μg of a ginsenoside Rg_1 or glucose was injected and the response was determined. The reaction time was calculated from the flow rate of the eluent and the internal volume of the capillary reaction coil. The internal volume of the reaction coil was calculated from the difference of the retention times of the salicylic acid with and without the reaction coil.

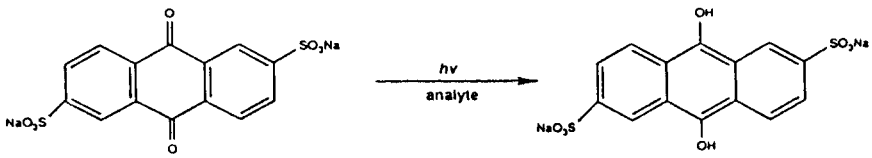
Analysis of ginseng saponins

Methanolic extract of white ginseng (3g) was extracted with *n*-butanol. The butanolic fraction was evaporated to dryness and the residue was dissolved in 50 m*l* of water. The solution was filtered through 0.45 μm membrane filter and applied to HPLC of which the operating condition is described above. The peaks were identified by co-injection of the standard ginsenosides.

RESULTS AND DISCUSSION

Two chromatographic systems were examined to optimize the analytical conditions. One is single pump system with reverse phase column, and AQDS was added to the eluent in this system (Fig. 2). The other is dual pump system with NH_2 column, and AQDS pumped by seondary pump was mixed to the column effluent at post-column (Fig. 3).

Single pump system was used to find the optimal concentration of AQDS and photoreaction time. Fig. 4 shows the effect of AQDS concentration in the eluent on the signal intensity. The signal intensity of ginsenoside Rg_1 and glucose increased until



anthraquinone-2,6-disulfonate dihydroxyanthracene-2,6-disulfonate

- Mechanism -

$Q + h\nu \longrightarrow Q^*$ (singlet,S)

$Q^*(S) \longrightarrow Q^*$ (triplet,T)

$Q^*(T) + CH_3CH_2OH \longrightarrow QH\cdot + CH_3\dot{C}HOH$

$CH_3\dot{C}HOH + Q \longrightarrow QH\cdot + CH_3CHO$

$2QH\cdot \longrightarrow Q + QH_2$

Q : anthraquinone-2,6-disulfonate

QH_2 : dihydroxyanthracene-2,6-disulfonate

Fig. 1. Principle of photoreduction fluorescence detection method.

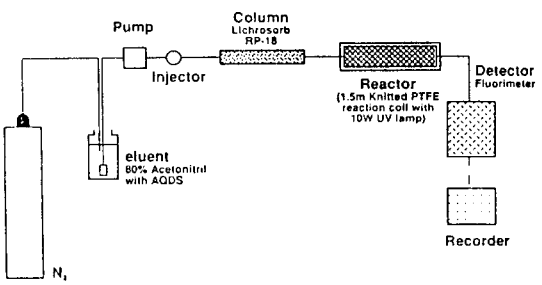


Fig. 2. Scheme of photoreduction fluorescence detection system (mono pump system).

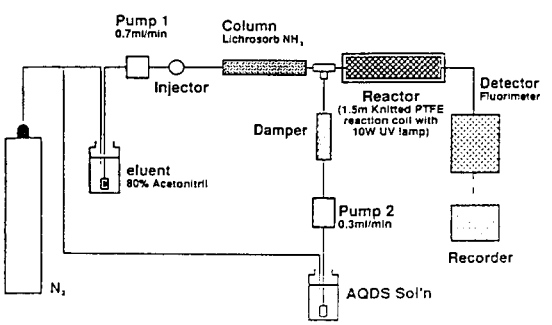


Fig. 3. Scheme of photoreduction fluorescence detection system (dual pump system).

the concentration of AQDS reaches to 1.0×10^{-3}M, showed the maximum at 1.0×10^{-3}M, and then decreased over this concentration. However, the

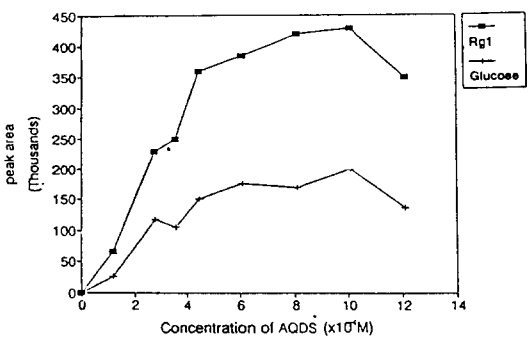


Fig. 4. The effect of AQDS concentration to the peak area.

10 W UV with 1.5 m PTFE tubing, Lichrosorb RP-18 (250 nm×4 mm i.d., 7 μm); Eluent: acetonitrile/water (80/20) with various concentrations of AQDS; Flow rate: 1 ml/min, reaction time: 8 sec; Detection: fluorescence (λ_{ex}: 400 nm, λ_{em}: 525 nm).

background noise continously increased to the concentration of AQDS, and at high concentration, it was difficult to detect them. Eventually, the optimum concentration of AQDS passing through the reactor was found to be 3.0×10^{-4}M.

Fig. 5 shows the effect of photoreaction time to the signal intensity. Photoraction time was changed by varying the flow rate of the eluent from 0.25 ml/min (reaction time : 32 sec) to 4 ml/min (reaction time : 2 sec). Reaction time 0 sec means the absence

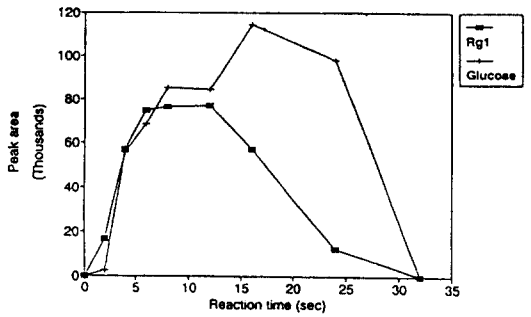


Fig. 5. The effect of reaction time on the peak area. Eluent: acetonitrile/water (60/20) with 3.0×10^{-4} M AQDS, HPLC condition is as in Fig. 4.

of photoreactor. The signal response of ginsenoside Rg_1 showed a curve similar to a parabola whose plateau ceiling appeared at 6-12 sec as shown in Fig. 5. The signal response of glucose also showed

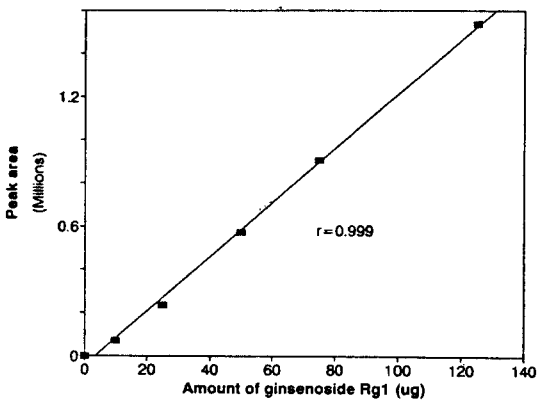


Fig. 6. Calibration curve of ginsenoside Rg_1. Column: Lichrosorb NH_2 (250 nm×4 mm i.d., 5 μm); Eluent: acetonitrile/water (80/20), 0.7 ml/min; 1.0×10^{-3}M AQDS was mixed to the column effluent (0.3 ml/min); Detection: Fluorescence (λ_{ex}: 400 nm, λ_{em}: 525 nm).

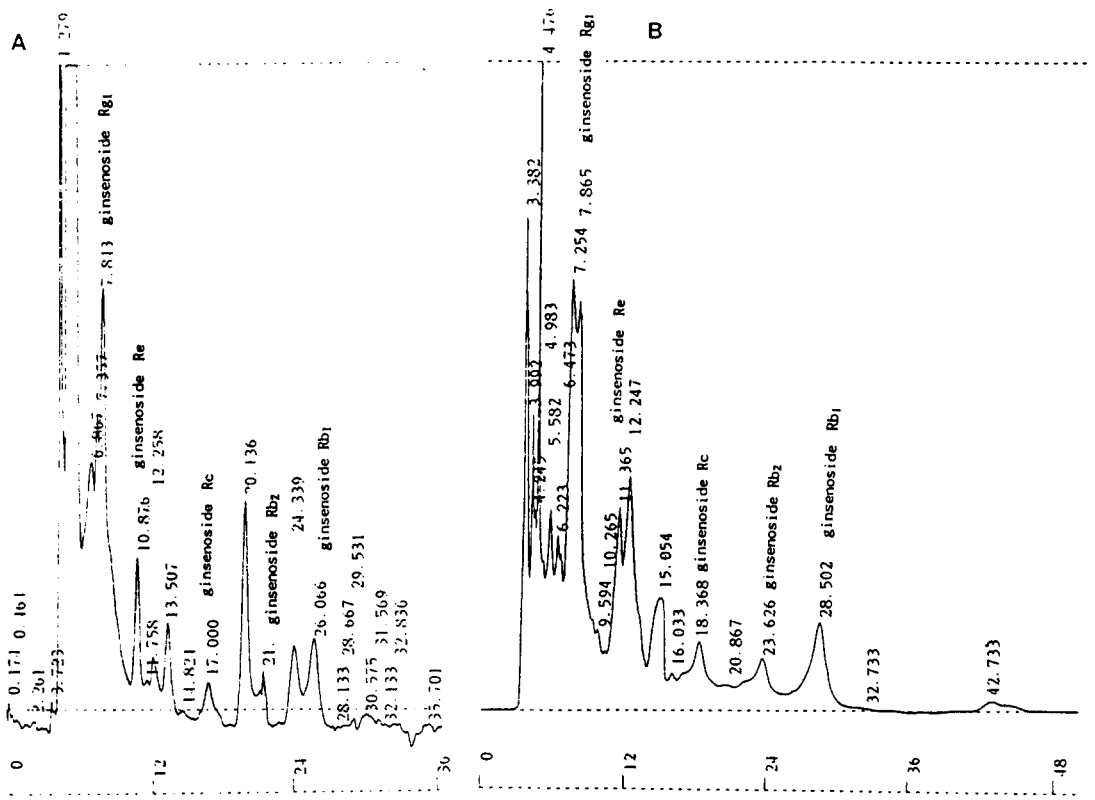


Fig. 7. Chromatogram of white ginseng: photoreaction (A) and UV (B) detection. Condition of A: as in Fig. 6; Condition of B: Lichrosorb NH_2 (250 nm×4 mm i.d., 5 μm), acetonitrile/water (80/20), 0.7 ml/min, UV 203 nm.

a parabolic curve but its maximum appeared at 16 sec. Therefore, the flow rate was adjusted to 1.0 ml/min in the reactor and the length of PTFE capillary was fixed to 1.5 m. The photoreaction time was 8 sec at this condition.

The calibration curve of ginsenoside Rg_1 is shown in Fig. 6. The correlation coefficient of the calibration curve of this method was 0.999 and the dynamic linear range was *ca.* 10^2 from 1 μg to 100 μg. The detection limit of ginsenoside Rg_1 is *ca.* 350 ng at a signal-to-noise ratio of 3 : 1. Photoreaction fluorescence detection method showed better sensitivity than RI detection method, but somewhat poorer sensitivity than UV detection at 203 nm. However, this detection mehod showed a good selectivity for the ginsenosides. For example, the peaks which appeared near ginsenoside Rg_1 and Re in UV detection method (chromatogram B in Fig. 7) were decreased or not appeared in photoreaction method (chromatogram A in Fig. 7).

Consequently, we think that the photoreduction-fluorescence detection method described herein is applicable to the analysis of ginsenosides in drug preparations which are diffficult to apply HPLC/UV or RI detection method because of interferences. Further study to increase the sensitivity and the selectivity of this method is in progress.

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