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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR A WIDE RANGE OF NATURALLY OCCURRING GLYCOSIDES

HITOMI KAIZUKA and KUNIO TAKAHASHI*
Meiji College of Pharmacy Nozawa 1-35-23, Setagaya-ku, Tokyo 154 (Japan)
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SUMMARY

A high-performance liquid chromatographic method using a water-containing silica gel column and water-containing solvent systems was developed for the separation of water-soluble glycosides including saponins. The method can be applied efficiently to both analytical and preparative procedures.

INTRODUCTION

Recently, various methods have been developed for the high-performance liquid chromatographic (HPLC) analysis of naturally occurring glycosides, among which reversed-phase columns (ODS and amino) have frequently been used[1–10]. However, reversed-phase HPLC takes time for the separation of complex mixtures of natural products and can not be used directly for their preparative separation.

We have attempted to improve the HPLC separation of water-soluble glycosides by using a normal silica gel column but with a slight modification in the packing process. Similar lines of work using silica gel or a diatomaceous earth column with a mobile phase containing a small amount of water were reported earlier by other workers[8,11–15] for the separations of carbohydrates, triterpenes and DNA.

Our method, using a specially packed silica gel column and efficient solvent systems, can be applied successfully for both analytical and preparative purposes without the need for any pretreatment and with a short operation time.

In the present paper, the separations of triterpenoid saponins, cardiac glycosides and monoterpenoid and flavonoid glycosides are demonstrated as applications of this method.

EXPERIMENTAL

Preparation of column

Analytical silica gel column. After a column had been prepared with Nucleosil 50-5 (Macherey, Nagel & Co., Düren, G.F.R.) by a normal slurry method, methanol was passed through at a rate of 12 ml/min for 20 min at a pressure of 400–450 kg/cm^2, followed by a solvent mixture [chloroform–methanol–ethanol–water (62:16:16:6)] at

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the same rate for 30 min. Before use the column was conditioned with the most appropriate solvent for 30 min. A typical column had dimensions 25 × 4.6 mm.

Semi-preparative silica gel column. The basic process for the preparation of columns was performed by the method above, except that various modifications were needed for scaling up. The flow-rate of the solvents and the pressure were increased to 30 ml/min and 120–150 kg/cm^2, respectively.

Preparative silica gel column. Methanol was passed through a Waters Prep-500 silica column at a rate of 50 ml/min for 15 min, and the solvent was then run through the column for 20 min before use.

All the solvents employed were HPLC grade.

Instruments

For large-scale preparation. A Waters Associates System 500A high-performance liquid chromatograph with a Prep-500 silica column was used for the large-scale separation of glycoside mixtures.

For semi-preparative and analytical procedures. A high-performance liquid chromatograph consisting of an SP-800-150-DX (Senshu Scientific Co., Tokyo, Japan) solvent pumping system with a Bellows damper (NBD-III), a Shodex SE-11 (Showa Denko K.K., Tokyo, Japan) RI detector, SSC-Y-1000 (Sensyu Scientific Co.) UV detector and a Rheodyne Model 7125 injection valve was used. Chromatograms were recorded on a Rikadenki Model R-201 recorder. The experiments were performed on the columns prepared as mentioned above. Columns employed were Sensyu-Pak Aquasil SS-452N (25 cm × 4.6 mm I.D.) and SS-762N (30 cm × 20 mm I.D.) (Sensyu Scientific Co.).

Mobile phase

The solvent systems used for the separations contained a small amount of water. The volumetric ratios of each component are shown in Table I.

Extraction

All plant materials were extracted with hot methanol. The butanol fractions of ginseng and bupleurum extracts were prepared by a known method.

Authentic samples

All the compounds separated by the present HPLC experiments were identified by comparison with authentic samples.

TABLE I

COMPONENTS AND THEIR VOLUMETRIC RATIOS IN MOBILE SOLVENT SYSTEMS

Solvent system	*Chloroform*	*Dichloromethane*	*Methanol*	*Ethanol*	*Water*
Solvent A	30		17		2
Solvent B	62		16	16	6
Solvent C	30		10		1
Solvent D	90		10		1
Solvent E		84	15		1.5

RESULTS AND DISCUSSION

An HPLC procedure using a silica gel column and a solvent system containing a small amount of water was used for the separation of water-soluble plant products, such as the saponins of ginseng and bupleurum roots, the glycosides of paeony and pueraria roots, and the bitter principles of *Swertia japonica*, as well as the cardiac glycosides of *Digitalis*.

Ginseng saponins

The butanol fraction of ginseng [the root of *Panax ginseng* (Araliaceae)] extracts contained several triterpenoid saponins named ginsenosides R_x(x = 0, a_1, a_2, b_1, b_2, c, d, e, f, g_1, g_2, etc.). Various HPLC studies on qualitative and quantitative analyses of ginsenosides R_x in ginseng have been reported, mostly using reversed-phase systems[1,2].

In the present study, a large-scale separation of extracts of ginseng was performed successfully using a Waters System 500A chromatograph with a specially treated Prep PAK-500/silica column (30 cm × 50 mm I.D.) as shown in Fig. 1. It demonstrated that the method can be applied efficiently to large-scale preparations of the individual saponin components.

A semi-preparative separation of saponins on an HPLC column (30 cm × 20 mm I.D.) was achieved by single injection of the extracts. A 200-mg volume of the ginseng extracts was applied to the column to separate the ginsenosides, almost all of which, except R_{g-1}, showed a single spot on thin-layer chromatography (TLC). The yield of ginsenoside R_e, for example, was 4.2 mg (Fig. 2.). This semi-preparative

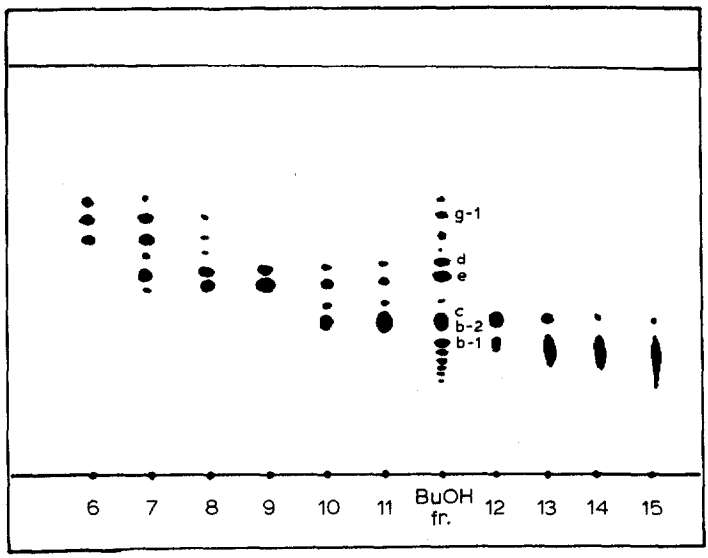

Fig. 1. TLC pattern of each fraction separated from the butanol (BuOH) fraction of ginseng on a Waters System 500A preparative HPLC system. For HPTLC: LHP-KF (Whatman); developing solvent, chloroform–methanol–water (65:35:10, lower layer); detection, sulphuric acid method. For HPLC: column, Prep 500 silica washed with methanol; eluent, solvent B; each fraction, 200 ml; flow-rate, 50 ml/min.

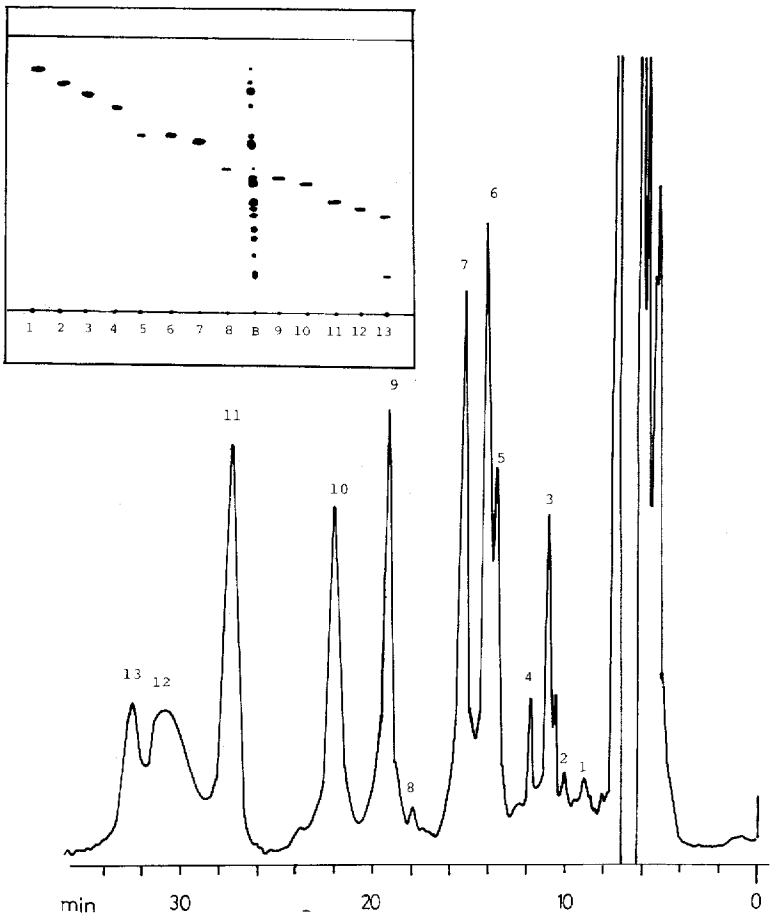

Fig. 2. HPLC profile and TLC pattern of the butanol fraction of ginseng (semi-preparative procedure). For HPTLC: LHP-KF; developing solvent: chloroform–methanol–water (65:35:10, lower layer); detection: sulphuric acid method. For HPLC: column, Aquasil SS-762N (30 cm × 20 mm I.D.); eluent, solvent A; flow-rate: 11 ml/min; detector: refractive index (RI); charged amount: 200 mg per 0.5 ml of methanol. Ginsenosides R_x: 3 = g-1; 6 = d; 7 = e; 9 = c; 10 = b-2; 11 = b-1. B = butanol fraction of ginseng extract.

method can be used successfully for the investigation of other saponins from biological materials.

HPLC analysis using a column of dimensions 25 cm × 4.6 mm I.D. can be applied to the efficient quantification of herbal drugs. The extracts of ginseng and its botanical congeners were analysed by several types of chromatography for the purposes of identification and quantification.

The present HPLC procedure gave excellent results in the chemical analysis of ginseng and its congeners, such as American ginseng and Sanchi ginseng, as shown in Figs. 3–5.

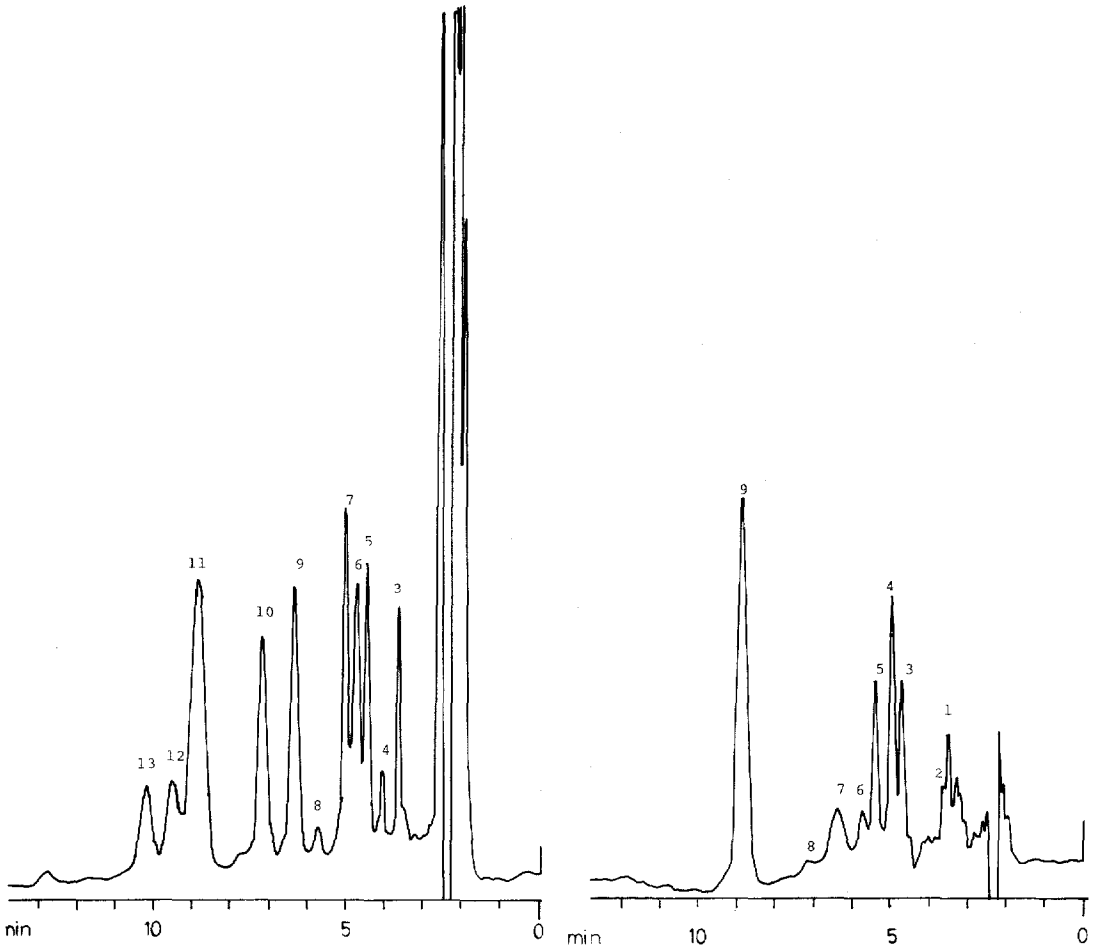

Fig. 3. HPLC profile of the butanol fraction of ginseng. Column: Aquasil SS-452N (30 cm × 4.6 mm I.D.). Eluent: solvent A. Flow-rate: 1.0 ml/min. Detector: RI. Ginsenosides R_x: 3 = g-1; 5 = d; 7 = e; 9 = c; 10 = b-2; 11 = b-1.

Fig. 4. HPLC profile of the butanol fraction of American ginseng. Column: Aquasil SS-452N. Eluent: solvent A. Flow-rate: 1.6 ml/min. Detector: RI. Ginsenosides R_x: 3 = d; 4 = e; 7 = c; 9 = b-1.

Saponins of Bupleurum root

Saikosaponins were isolated from the root of *Bupleurum falcatum* and related species (Umbelliferae); these have long been used as an important drug [Chai-fu (Saiko)] in traditional Chinese medicine.

Saikosaponins a and d show anti-inflammatory and anti-hepatotoxic activities, whereas saikosaponin c is ineffective. Thus, HPLC analysis of these saponins is practically useful, and the present procedure was applied very efficiently for both analytical and semi-preparative purposes, as shown in Figs. 6–9.

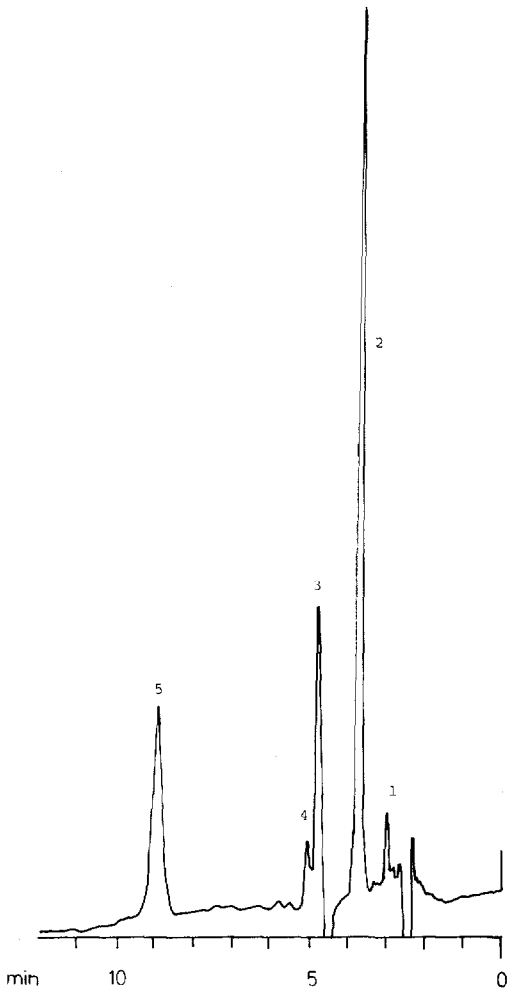

Fig. 5. HPLC profile of the butanol fraction of San-chi ginseng. Column: Aquasil SS-452N. Eluent: solvent A. Detector: RI. Ginsenosides R_x: 1 = g-1; 3 = d; 5 = b-1.

Cardiac glycosides of Digitalis

The steroidal glycosides of Digitalis leaves are well known as useful heart drugs. The preparative-scale separation and the chemical analysis of these cardiac glycosides are of practical importance, and the present procedure gave good results (Fig. 10).

Flavonoid glycosides of Pueraria root

A methanol extract of *Pueraria lobata* containing isoflavone glycosides such as puerarin, daidzin and PG-3 was subjected to a large-scale separation by the present procedure. From the HPLC eluates were isolated puerarin and daidzin in their pure states by treating the residue after evaporation of the fractions with butanol and methanol, respectively. The same procedure was also adopted for analytical-scale HPLC (Fig. 11).

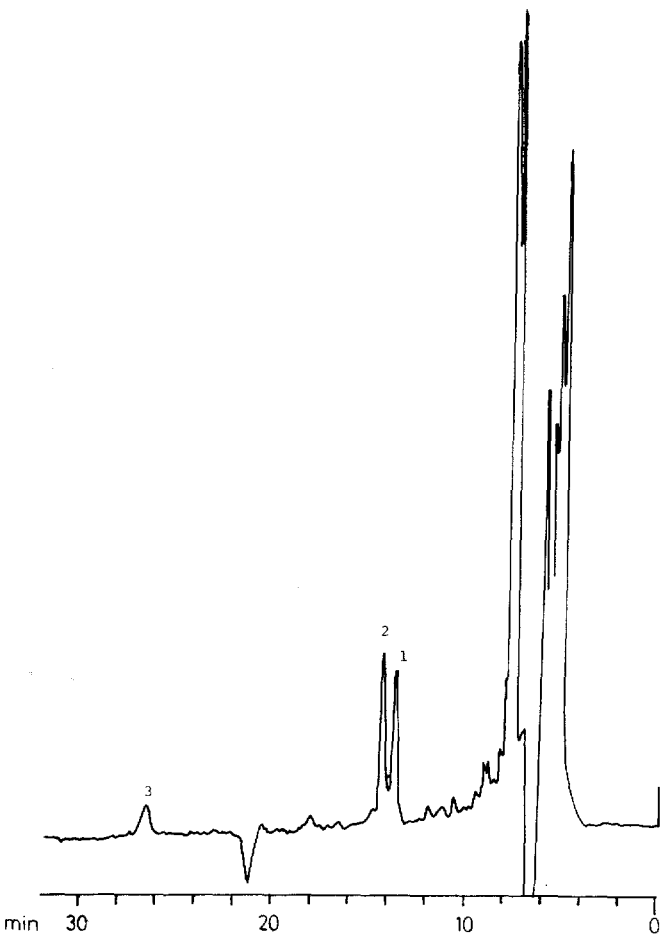

Fig. 6. HPLC profile of the butanol fraction of *Bupleurum falcatum* root. Column: Aquasil SS-762N (30 cm × 20 mm I.D.). Eluent: solvent C. Flow-rate: 11 ml/min. Detector: RI. Saikosaponins: 1 = d; 2 = a; 3 = c.

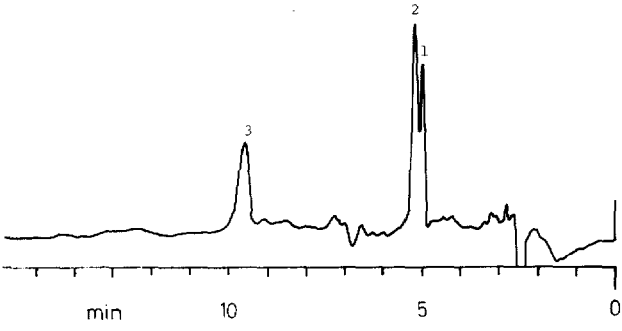

Fig. 7. HPLC profile of the butanol fraction of *Bupleurum longeradiatum* root. Column: Aquasil SS-452N (30 cm × 4.6 mm I.D.). Eluent: solvent C. Flow-rate: 1.0 ml/min. Detector: RI. Saikosaponins: 1 = d; 2 = a; 3 = c.

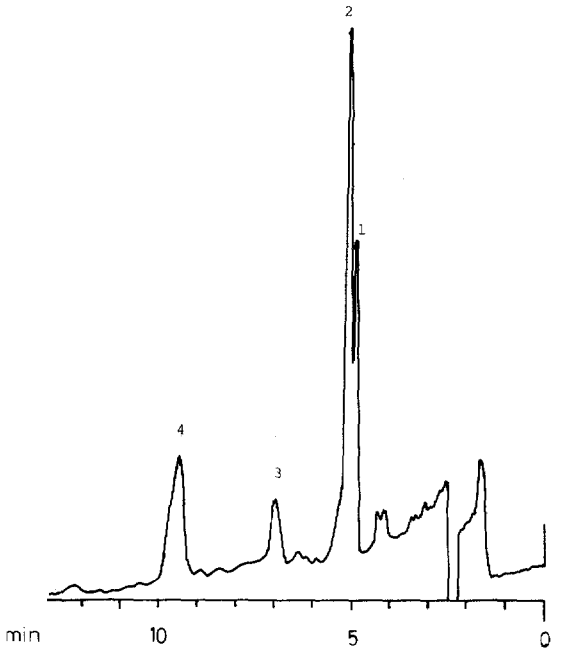

Fig. 8. The butanol fraction of *Bupleurum* root purchased from a Chinese drug market. Column: Aquasil SS-452N. Eluent: solvent C. Flow-rate: 1 ml/min. Detector: RI. Saikosaponins: 1 = d; 2 = a; 4 = c.

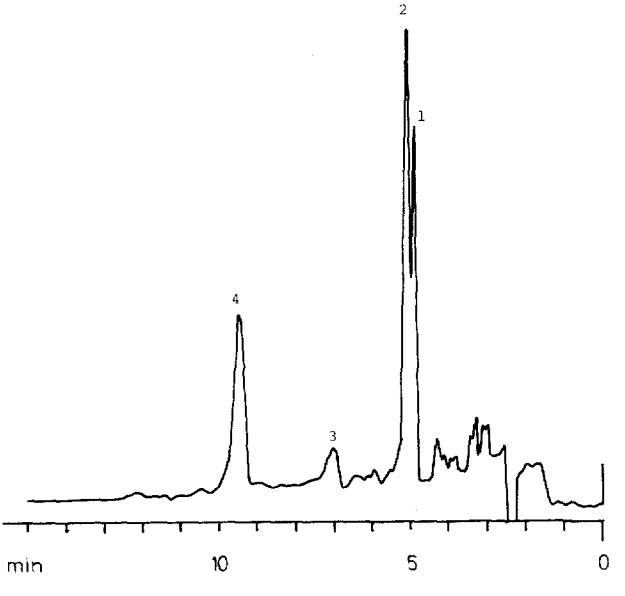

Fig. 9. HPLC profile of the butanol fraction of *B. falcatum* root. Column: Aquasil SS-452N. Eluent: solvent C. Flow-rate: 1 ml/min. Detector: RI. Saikosaponins: 1 = d; 2 = a; 4 = c.

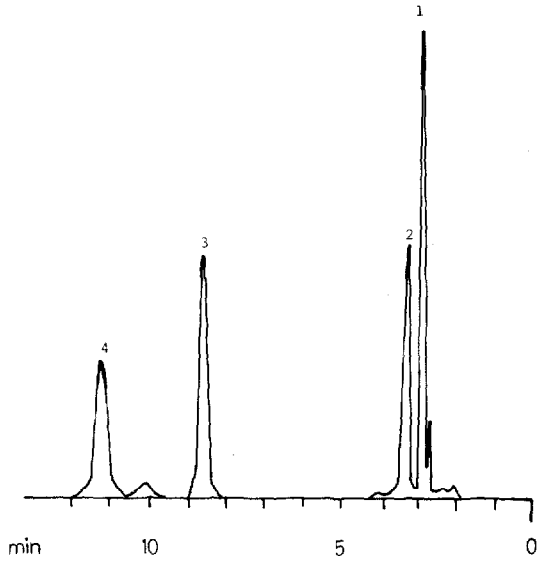

Fig. 10. Separation of Digitalis cardiac glycosides on HPLC. Column: Aquasil SS-452N. Eluent: solvent F. Flow-rate: 1.3 ml/min. Detector: UV 240 nm. Peaks: 1 = digitoxin; 2 = gitoxin; 3 = purpurea glycoside A; 4 = purpurea glycoside B.

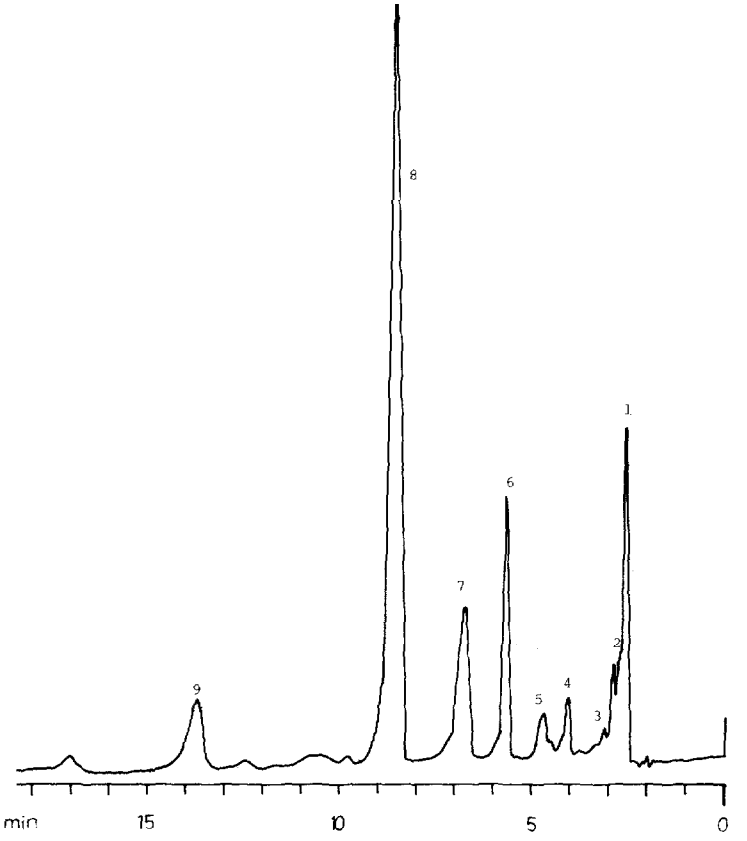

Fig. 11. HPLC profile of the methanol extract of *Pueraria lobata* root. Column: Aquasil 452N. Eluent solvent B. Flow-rate: 1 ml/min. Detector: UV 280 nm. Peaks: 6 = Daidzin; 7 = PG-3; 8 = puerarin.

Monoterpene glycosides of Paeony root and Swertia herbs

By the present HPLC method, good results were obtained in the separation of monoterpene glycosides from methanolic extracts of the root of *Paeonia albiflora* (Paeoniaceae) (Fig. 12) and the whole plant of *Swertia japonica* (Gentianaceae) (Fig. 13).

CONCLUSIONS

The advantages of the present HPLC method for the efficient separation of water-soluble glycosides can be summarized as follows.

(1) Constant and reasonable retention times are recorded even after prolonged use of the column.

(2) High solubilities in the present solvent system show the suitability of the procedure for water-soluble compounds.

(3) Excellent baseline separations are obtained in both analytical and preparative experiments.

(4) Excellent separations with less tailings of peaks are achieved without using gradient elution systems.

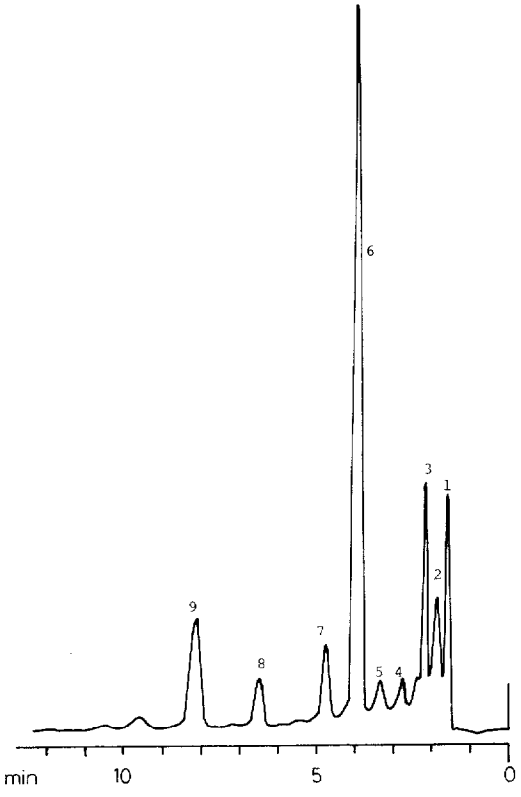

Fig. 12. HPLC profile of the methanol extract of *Paeonia albiflora* root. Column: Aquasil SS-452N. Eluent: solvent B. Flow-rate: 1.0 ml/min. Detector: UV 254 nm. Peaks: 2 = benzoylpaeoniflorin; 6 = paeoniflorin; 9 = oxypaeoniflorin.

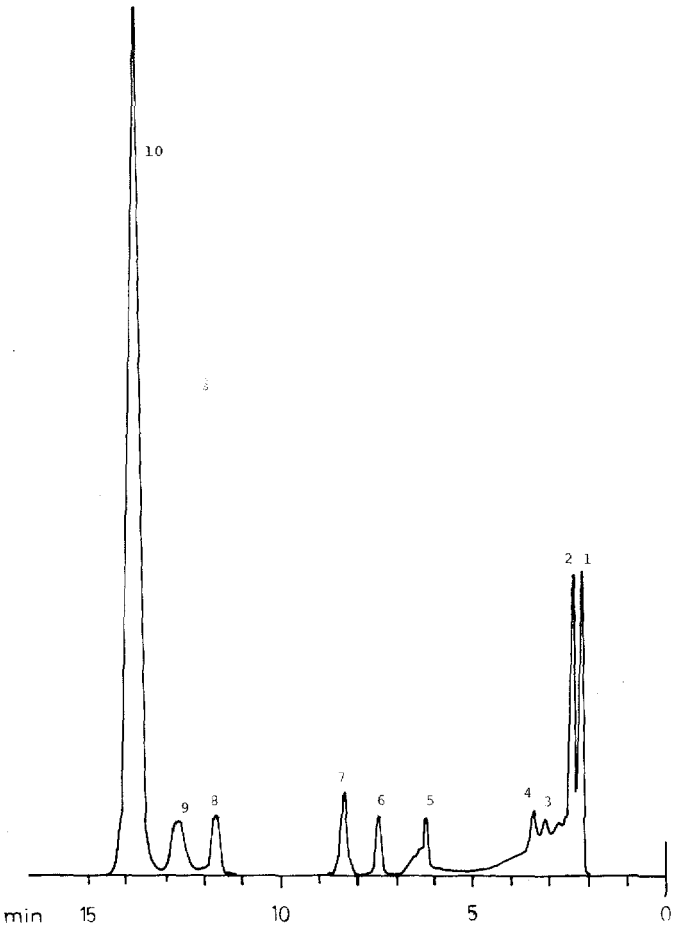

Fig. 13. HPLC profile of the methanol extract of *Swertia japonica* (whole plant). Column: Aquasil SS-452N. Eluent: solvent D. Flow-rate: 1 ml/min. Detector: UV 240 nm. Peaks: 5 = amarogentin; 6 = amaroswerin; 9 = sweroside; 10 = swetiamarin.

(5) Lower operating pressures are needed compared with ODS systems.

(6) There is a wide availability of solvent systems for the mobile phase and it is easy to select the optimum mobile phase by performing TLC on silica gel and checking for R_F values in the range 0.2–0.8.

(7) Parallel correlations exist between the elution sequences of the compounds on HPLC and their R_F values on TLC when using the same solvent systems.

(8) The HPLC columns are easily regenerated by washing with methanol and then passing through suitable solvents.

(9) The columns are much cheaper compared with the usual reversed-phase columns.

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