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Note

High-performance liquid chromatographic analysis of naturally occurring glycosides and saponins

TORU OKUYAMA*, MITSUMASA TAKATA and KUNIO TAKAHASHI
Department of Pharmacognosy and Phytochemistry, Meiji College of Pharmacy, 1-35-23 Nozawa, Setagaya-ku, Tokyo 154 (Japan)
and
TOSHIHIRO ISHIKAWA, KENNJI MIYASAKA and NAOKI KANEYAMA
Toa Nenryo Kogyo Co. Ltd., 1-1-1 Hitotsubashi, Chiyoda-ku, Tokyo 100 (Japan)
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Reversed-phase high-performance liquid chromatography (HPLC) has been efficiently applied to the separation of glycosides. We have already demonstrated that an Aquasil silica gel column with an aqueous mobile phase was effective for the separation of water-soluble glycosides by HPLC[1]. It was efficiently applied to the separation of Ginseng saponins, Bupleurum saponins, Cardiac glycosides, Paeony glycosides, Swertia herb glycosides and Pueraria glycosides. Recently, a hydroxyapatite column has been developed for the HPLC separation and purification of a wide variety of peptides, proteins, enzymes and nucleic acids[2–6]. Using this HPLC column, Kasai *et al.*[7] first demonstrated the efficient separation of glycosides and sugars. This paper describes the application of this column to the separation of monoterpene, cyanogenic and coumarin glycosides and saponins.

EXPERIMENTAL

Plant materials

The following were used: Bupleuri Radix (*Bupleurum falcatum* Linne) (Umbelliferae); *Bupleurum* root, purchased on the Chinese drug market; *B. longiradiatum* Turcz., collected in the medicinal plant garden of the high-coldish ground at Yatsugatake, Meiji College of Pharmacy; Zi-Hua Qian-Hu [*Peucedanum decursivum* Maxim. (= *Angelica decursiva* Fr. et Sav.) (Umbelliferae)]; Bai-Hua Qian-Hu [*P. praeruptorum* Dunn.]; Persicae Semen (*Prunus persica* Batsch) (Rosaceae) and Paeoniae Radix (*Paeonia lactiflora* Pallas) (Paeoniaceae).

Extraction

The pulverized plant material (5 g) was extracted with 50 ml of hot methanol and the concentrated extract was loaded on an Amberlite XAD-2 (1 g) column and eluted twice with methanol to obtain a glycoside fraction. The residue after evaporation was dissolved in 100 μl of methanol and a 5-μl aliquot was injected to HPLC.

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Apparatus and conditions for HPLC

The HPLC instrument consisted of a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), SP-8700XR solvent delivery system (Spectra-Physics, San Jose, CA, U.S.A.), Y-1000 UV detector (Senshu Scientific, Tokyo, Japan) and SIC-7000A integrator equipped with an 8-in. disk drive system (System Instruments, Hachioji City, Tokyo, Japan).

A TPS-326010 HPLC column (100 × 7.5 mm I.D.) packed with hydroxyapatite (2.2 μm) (Toanenryo Kogyo, Tokyo, Japan) and an Aquasil SS 452N column (250 × 4.5 mm I.D.) packed with aqueous silica gel (5 μm) (Senshu Scientific) were used.

The volumetric ratios of the components of the mobile phase and the linear gradient used are shown in Table I. All solvents were of HPLC grade (Nakarai, Kyoto, Japan).

TABLE I
MOBILE PHASE COMPOSITIONS

Column	*Solvent system*	*Gradient*				
		Initial state		*Final state*		*Time (min)*
		Acetonitrile	*Water*	*Acetonitrile*	*Water*	
Hydroxyapatite	A	90	10	70	30	120
	B	90	10	50	50	60
	C	95	5	85	15	60
	D	95	5	65	35	60
	E	95	5	75	25	60
		Isocratic				
		Chloroform	*Methanol*	*Water*		
Aqueous silica gel	F	60	12	1		
	G	30	10	1		

Authentic samples

All the components separated were identified by comparison with authentic samples. The authentic sample of amygdalin was a commercial product (Nakarai) and all others were isolated as described previously[8–11].

RESULTS AND DISCUSSION

The geometric location of adsorbing groups, *e.g.*, carboxyl, phosphate, amino and guanidinyl groups, on the local molecular surface of macromolecules would fit the active surface of hydroxyapatite, but the adsorbing mechanism for lower molecular mass organic compounds has not fully been elucidated.

HPLC on a hydroxyapatite column has now been used for the separation of hydrophilic plant glycosides, such as the saponins of Bupleuri Radix and Zi-Hua

Qian-Hu, the cyanogenic glycosides of peach kernel, the monoterpene glycosides of paeony root and the coumarin glycosides of Qian-Hu, Zi-Hua Qian-Hu and Bai-Hua Qian-Hu.

Saponins of Bupleurum root

The extract of *Bupleurum falcatum* contained three major triterpenoid saponins, named saikosaponin a, c and d. Kaizuka and Takahashi[1] previously reported the separation of saikosaponins using an Aquasil column. The present HPLC system on hydroxyapatite also gave excellent results, and the different *Bupleurum* species corresponded to the elution profies of saikosaponins, as shown in Fig. 1.

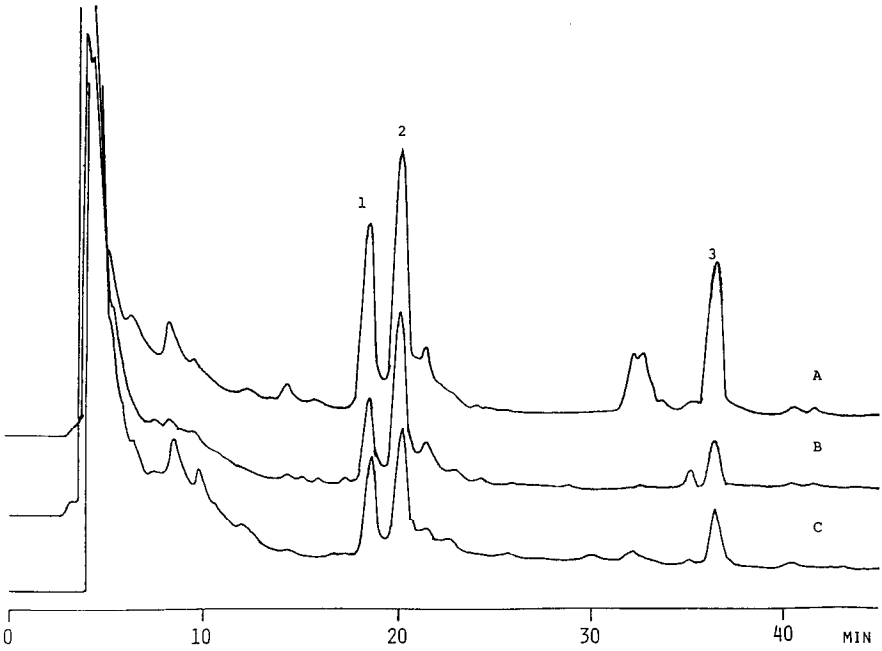


Fig. 1. HPLC profiles of XAD-2 fractions of Bupleuri Radix. (A) *B. falcatum*; (B) roots purchased on the Chinese drug market; (C) *B. longiradiatum*. Column, hydroxyapatite; eluent, solvent A (Table I); flow-rate, 1.0 ml/min; detector, UV (205 nm). Saikosaponins: 1 = d; 2 = a; 3 = c.

Saponins of Zi-Hua Qian-Hu

We isolated five bisdesmoside saponins having an oleanane skeleton as the sapogenin hederagenin from Zi-Hua Qian-Hu, as shown in Fig. 2[12]. Among these saponins Pd saponin V, 3-arabinopyranosyl-28-gentiobiosylhederagenin, was identified with an authentic sample isolated from *Akebia quinata* by Higuchi and Kawasaki[13]. The structural elucidation of the other four new saponins will be reported elsewhere. Saponins of higher molecular mass (over 1000) could not be separated well by Aquasil, whereas a good separation was achieved by using the hydroxyapatite column. Longer retention times are given by the saponins that have longer sugar residues.

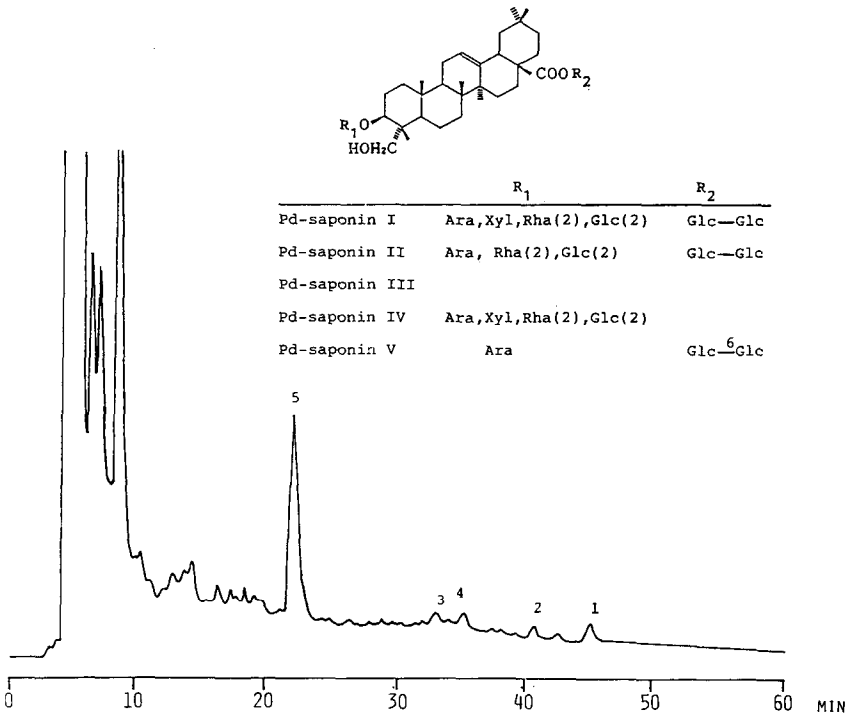


Fig. 2. HPLC profile of XAD-2 fraction of Zi-Hua Qian-Hu. Column, hydroxyapatite; eluent, solvent B (Table I); flow-rate, 1.0 ml/min; detector, UV (205 nm). Pd-saponins: 1 = V; 2 = III; 3 = IV; 4 = II; 5 = I.

Monoterpene glucosides of Paeoniae Radix

Paeoniflorin is the main glycosidic component of peony root. A good separation was obtained of the monoterpene glucoside, paeoniflorin and oxypaeoniflorin. The order of retention times was the same using both Aquasil and hydroxyapatite columns (Fig. 3).

Cyanogenic glucosides of peach kernel

Amygdalin, a cyanogenic glucoside, is a major component of peach kernel. Amygdalin is easily hydrolysed with emulsin to form benzaldehyde, hydrogen cyanide

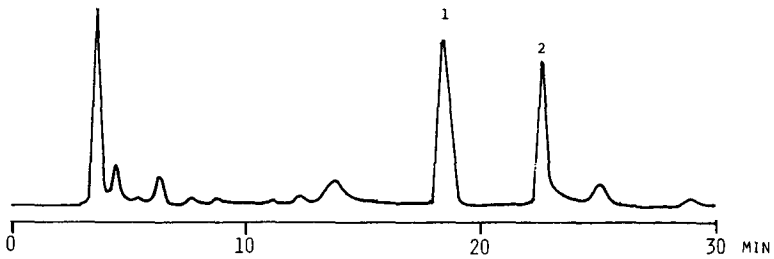


Fig. 3. HPLC profile of XAD-2 fraction of Paeoniae Radix. Column, hydroxyapatite; eluent, solvent C (Table I); flow-rate, 1.0 ml/min; detector, UV (254 nm). Peaks: 1 = paeoniflorin; 2 = oxypaeoniflorin.

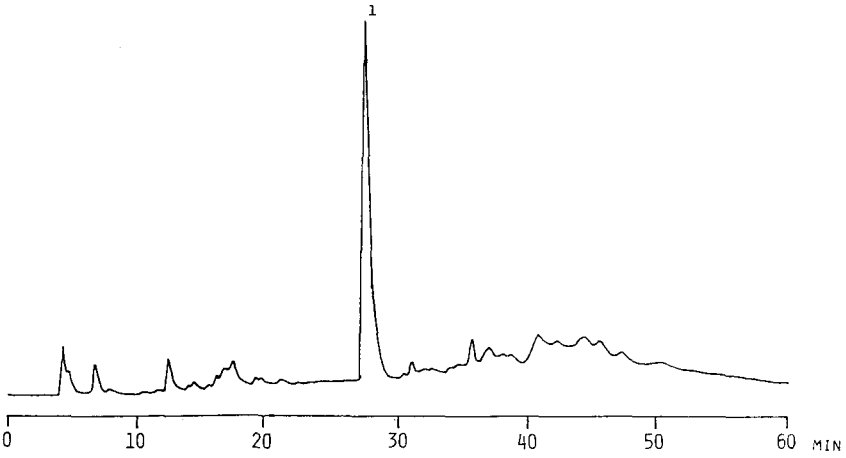


Fig. 4. HPLC profile of XAD-2 fraction of *Prunus persica* seed. Column, hydroxyapatite; eluent, solvent D (Table I); flow-rate, 1.0 ml/min; detector, UV (254 nm). Peak 1 = amygdalin.

and glucose. The odour of benzaldehyde is perceptible by grinding peach kernel with water. On the hydroxyapatite column, a good separation of amygdalin was achieved, as shown in Fig. 4.

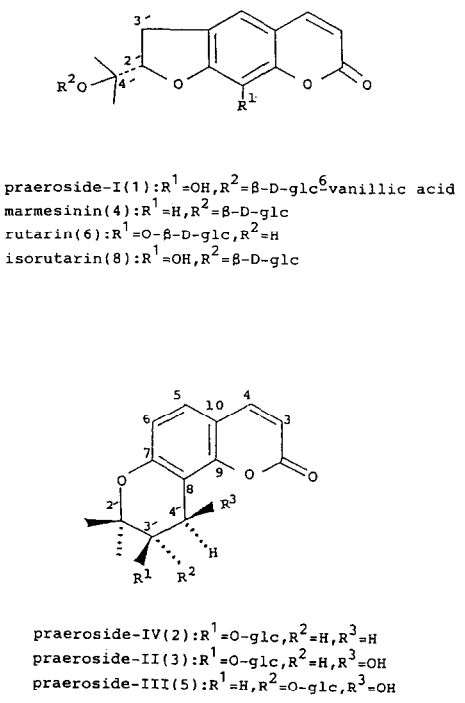


Fig. 5. Structures of coumarin glucosides from Bai-Hua Qian-Hu (Q-I type).

Coumarins of Qian-Hu

According to the ^1H NMR spectral data and the silica gel thin-layer chromatographic (TLC) densitometric profiles of the ethereal extracts, Qian-Hu on the drug market can be classified into four groups: Q-I, Q-II [both Chinese Bai-Hua series (*Peucadanum praeruptorum* root)], Q-III [including Chinese Zi-Hua series (*P. decursivum* root), identical with Japanese *Angelica decursiva* (Nodake root) and Q-IV (Korean *Anthriscus nemorosa* root)[14].

Coumarin glycosides of Bai-Hua Qian-Hu. From the *n*-butanol-soluble fraction of methanolic extracts derived from Bai-Hua Qian-Hu (Q-I type), the root of *P. praeruptorum*, four angular-type coumarin glycosides, praeroside II, III, IV and V, four linear-type coumarin glycosides, praeroside I, marmesinin, rutarin and isorutarin, and two simple coumarin glycosides, skimmin and scopolin, were isolated by using the Aquasil column and their structures[8,9] were established (Fig. 5).

The chromatography was performed using Aquasil or hydroxyapatite columns. The linear-type coumarin glycosides gave the same elution order on both columns. However, with the angular-type coumarin glycosides, the order of elution of praeroside II and III and also of skimmin and scopolin were the opposite on Aquasil and hydroxyapatite (Figs. 6 and 7).

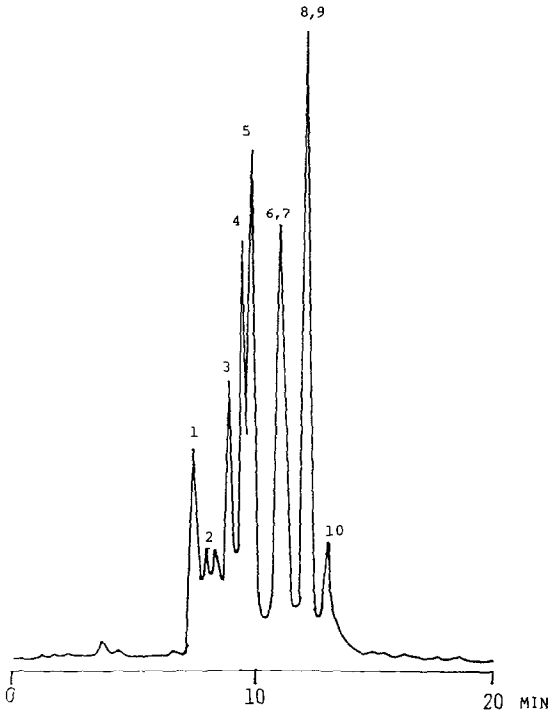


Fig. 6. HPLC profile of XAD-2 fraction of Bai-Hua Qian-Hu (Q-I type). Column, Aquasil; eluent, solvent F (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = praeroside I; 2 = preaeroside IV; 3 = paeroside II; 4 = marmesinin; 5 = praeroside III; 6 = rutarin; 7 = scopolin; 8 = isorutarin; 9 = skimmin; 10 = praeroside V.

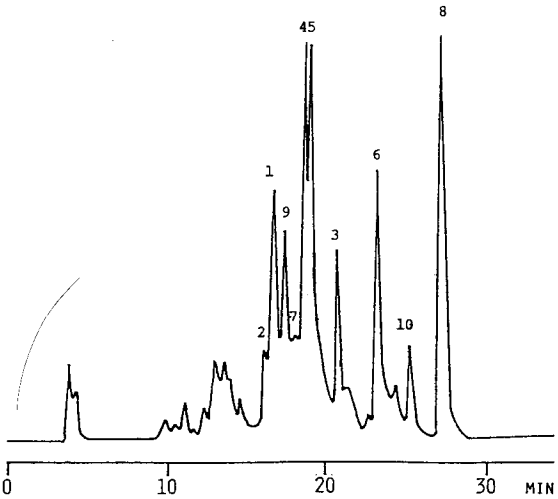
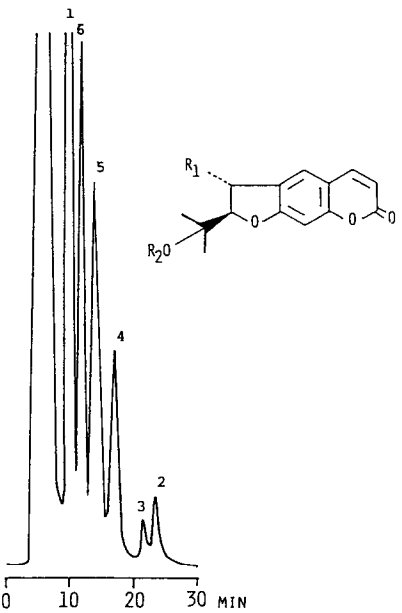


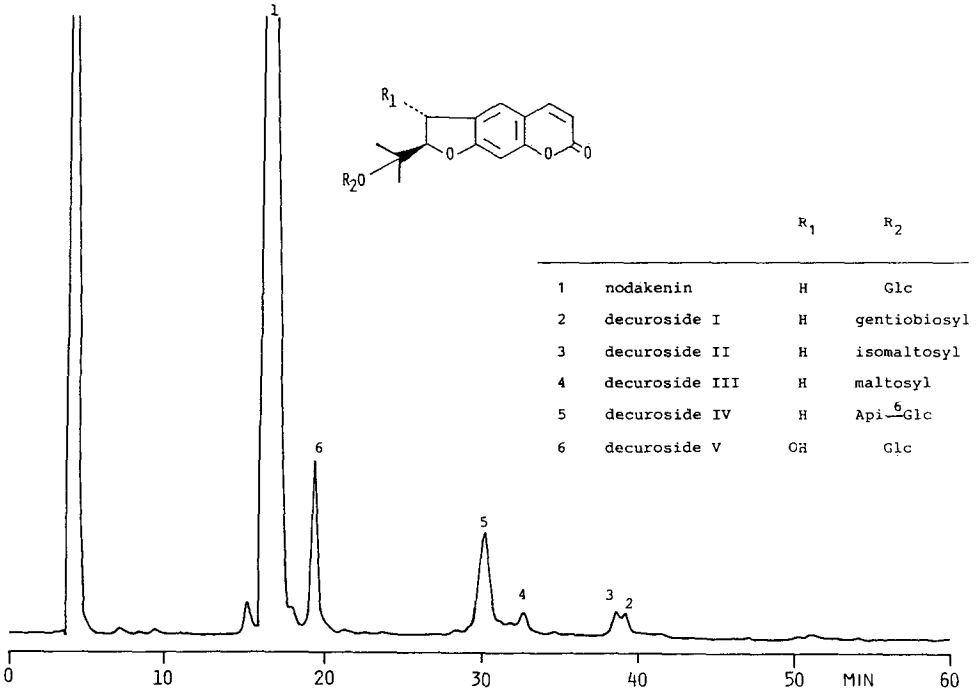
Fig. 7. HPLC profile of XAD-2 fraction of Bai-Hua Qian-Hu (Q-I type). Column, hydroxyapatite; eluent, solvent C (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = praeroside I; 2 = praeroside IV; 3 = praeroside II; 4 = marmesinin; 5 = praeroside III; 6 = rutarin; 7 = scopolin; 8 = isorutarin; 9 = skimmin; 10 = praeroside V.



		R_1	R_2
1	nodakenin	H	Glc
2	decuroside I	H	gentiobiosyl
3	decuroside II	H	isomaltosyl
4	decuroside III	H	maltosyl
5	decuroside IV	H	Api—^{6}Glc
6	decuroside V	OH	Glc

Fig. 8. HPLC profile of XAD-2 fraction of Zi-Hua Qian-Hu (Q-III type). Column, Aquasil; eluent, solvent G (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = nodakenin; 2 = decuroside I; 3 = decuroside II; 4 = decuroside III; 5 = decuroside IV; 6 = decuroside V.

Coumarin glycosides of Zi-Hua Qian-Hu. Six furocoumarin glycosides were isolated from Zi-Hua Qian-Hu, the root of *P. decursivum*[10,11], as shown in Fig. 8. Nodakenin was the main component of coumarin glycosides, and decuroside V was identical with 3′-hydroxynodakenin. Decuroside I–IV were differentiated according to their sugar moieties. An almost complete separation can be achieved using the Aquasil column (Fig. 8). Those having a disaccharide moiety including a branched sugar, apiose, had shorter retention times than those of glucobiosides. In the sugar linkage attached to the aglycone, nodakenin is different in decuroside I, II and III, and the order of retention times was III, II and I, which have α-(1–4)-, α-(1–6)- and β-(1–6)-diglucosyl moieties, respectively. The elution profile of the coumarin glycosides using a hydroxyapatite column was similar to that with an Aquasil column (Fig. 9). However, decuroside I and II could not be successfully separated on the hydroxyapatite column.



		R_1	R_2
1	nodakenin	H	Glc
2	decuroside I	H	gentiobiosyl
3	decuroside II	H	isomaltosyl
4	decuroside III	H	maltosyl
5	decuroside IV	H	Api$\frac{6}{}$Glc
6	decuroside V	OH	Glc

Fig. 9. HPLC profle of XAD-2 fraction of Zi-Hua Qian-Hu (Q-III type). Column, hydroxyapatite; eluent, solvent E (Table I); flow-rate, 2.0 ml/min; detector, UV (280 nm). Peaks: 1 = nodakenin; 2 = decuroside I; 3 = decuroside II; 4 = decuroside III; 5 = decuroside IV; 6 = decuroside V.

CONCLUSION

On the basis of these results, the advantages of the hydroxyapatite HPLC column can be summarized as follows. Excellent separations were achieved using a simple solvent system. Owing to the increase in the number of hydroxyl groups, the retention times increased. Concerning the sugar moieties, the diglucosal linkage shows

the order of retention times α-(1–4)- < α-(1–6)- < β-(1–6)-. The operating pressure required is lower than that in any other HPLC columns currently available. Parallel correlations exist between the elution sequence of the compounds in HPLC and their R_F values in silica gel TLC using chloroform–methanol–water as the solvent system. The HPLC column is easily regenerated by washing with 0.1 *M* sodium hydroxide solution. The column is widely applicable to the separation of naturally occurring glycosides, even high-molecular-mass compounds such as saponins.

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