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Note

High-performance liquid chromatographic analysis of β-escin

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β-Escin, the active constituent of *Aesculus hippocastanum* L., is a mixture of saponins derived from the triterpenes protoescigenin and barringtogenol[1] (Fig 1). Because of its antiinflammatory, antiedematous and capillaro-protective properties, β-escin is largely employed in the therapy of peripheral vascular disorders[2,3]. In recent years, it has found wide application also in the cosmetic field, mainly for the prevention/treatment of panniculopatia edemato-fibrosclerotica (so-called cellulitis)[4].

In spite of its widespread use, there have been few reports on the high-performance liquid chromatographic (HPLC) analysis of β-escin[5,6] and, due to the lack of standards, the assays described are based on determination of the aglycone

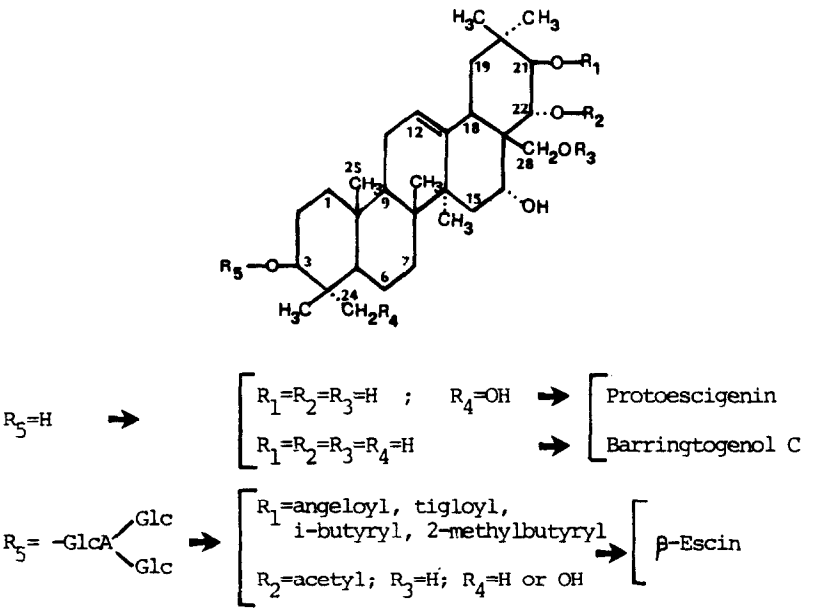

Fig. 1. Chemical structures of β-escin saponins. GlcA = Gluconic acid; Glc = glucose; i = iso.

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after alkaline hydrolysis or on relative peak areas. Furthermore, none of the reported methods offers a satisfactory separation of the components, to allow their isolation/characterization.

In this work we describe an efficient isocratic HPLC procedure for the isolation of the main saponin[7] 3-[2″-(β-D-glucopyranosido)-4′-(β-D-glucopyranosido)-β-D-glucuronopyranosido]-21-β-tigloyl-22-α-acetylprotoescigenin (I) and it 21-β-angeloyl analogue (II). These compounds have been used as reference standards for the analysis of β-escin samples.

EXPERIMENTAL

Materials

β-Escin was obtained from different commercial sources (Fluka-Schrepfer and Indena, Milan, Italy). Commercial samples were dissolved in water at a concentration of 0.3 mg/ml and centrifuged at 2500 *g* for 5 min to remove any particulate material. Acetonitrile and water were of HPLC grade (J. T. Baker, Deventer, The Netherlands).

Chromatographic conditions

The liquid chromatograph consisted of a Model U6K universal injector, a Model 510 pump, a Model Lambda Max 480 UV detector and a Model 990 photodiode array detector (Waters Assoc., Milford, MA, U.S.A.) connected to a CR3A integrator (Shimadzu, Kyoto, Japan).

Chromatographic experiments were performed on Spheri-5 RP-18 (100 mm × 4.6 mm, 5 μm; Brownlee Labs., Santa Clara, CA, U.S.A.) and on Microsorb 3-μm Spherical C_{18} (100 mm × 4.6 mm; Rainin, Woburn, MA, U.S.A.). To preserve the column life, precolumns (RP-18, 5 μm, OD-GU, Brownlee Labs.; Microsorb 3 μm C_{18}, guard No. 80-200-G3, Rainin) were used. The Rainin column and guard-column were supplied by Biolabo Instrument (Milan, Italy).

The mobile phase was acetonitrile–water–20% phosphoric acid (33.5:66.5:0.1), pH 3.2; the flow-rate was 1.0 ml/min. Samples (20 μl) were applied on the column and the peaks were monitored at 205 nm (a.u.f.s. = 0.032).

Isolation of saponins I and II

Aliquots (50 μl) of an aqueous solution of β-escin (2 mg/ml) were repetitively (four or five times) injected and eluted as described above. The major peaks [14.8 (I) and 18.1 (II) min] were collected by means of a Model 201 fraction collector (Gilson, Biolabo, Milan, Italy) and the corresponding fractions were dried under vacuum. Their purity was confirmed by rechromatography.

Mass spectrometry

Fast atom bombardment (FAB) mass spectra were obtained on a VG Analytical Model 70-70 EQ instrument, employing argon atoms with kinetic energy 7 keV. Recordings in the negative ion mode were taken at a resolution of 3000, with a speed of 20S/decade. Data were processed by a Digital PDP 8/A computer system. Matrix: thioglycerol.

RESULTS AND DISCUSSION

The purpose of the present investigation was to develop a simple and rapid HPLC procedure for the isolation of the main saponins (I and II) of β-escin. This required a determination of the chromatographic conditions suitable for a well resolved fingerprinting of β-escin under isocratic conditions. The Brownlee Labs. RP-18 and Microsorb C_{18} columns provided the best resolution in comparison with Bio-Rad C_{18} Nova-pak and Hypersil columns, so confirming the results obtained during a study of Ginseng saponins[8].

To avoid peak broadening due to the presence of carboxyl groups in the escin,

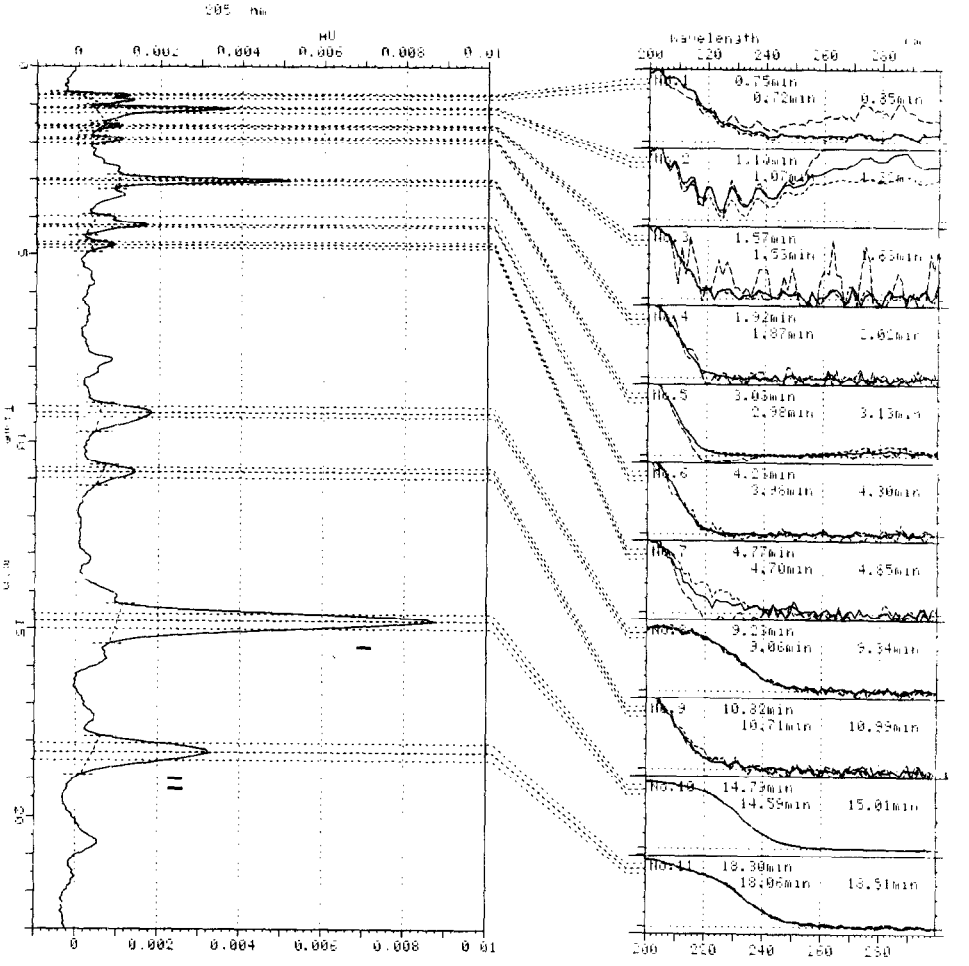

Fig. 2. Chomatogram of β-escin. Peaks: I = 3-[2″-(β-D-glucopyranosido)-4′-(β-D-glucopyranosido)-β-D-glucuronopyranosido]-21-β-tigloyl-22-α-acetylprotoescigenin; II = 3-[2″-(β-D-glucopyranosido)-4′-(β-D-glucopyranosido)-β-D-glucuronopyranosido]-21-β-angeloyl-22-α-acetylprotoescigenin. Eluent: acetonitrile–water 20%–phosphoric acid (33.5:66.5:0.1), pH 3.2; flow-rate, 1.0 ml/min. UV detection at 205 nm. On the right, the spectra of the ascending slopes (·····), the descending slopes (------) and the maxima of the various peaks are shown, together with the respective retention times.

the mobile phase was brought to acidic pH (3.2). Different percentages of acetonitrile and flow-rates were tested. A sharp separation was achieved with 33.5% acetonitrile in water (pH 3.2) over 20 min at a flow-rate of 1.0 ml/min (Fig. 2).

The main peak related to saponin I and the secondary peak related to saponin II were eluted with retention times of 14.8 and 18.1 min, respectively and they were easily collected automatically.

The negative ion FAB mass spectrum of saponin I (Fig. 3a) shows an abundant deprotoned molecular ion at *m/z* 1130 (the base peak) and fragment ions at *m/z* 1100 (which arises from the molecular ion by loss of a CH_2OH residue) and at *m/z* 1088, due to the loss of a $COCH_3$ residue. The other abundant fragment ion at *m/z* 968 corresponds to cleavage of the glycosidic bond accompanied by transfer of a hydrogen atom from the leaving sugar $[M-H-\text{glucose}]^-$. No significant fragment ions were detectable in the lower mass range of the spectrum.

Saponin II shows in the negative ion mode a deprotonated molecular ion at *m/z* 1130 (the base peak) and a fragmentation pattern perfectly superimposable on that of saponin I (Fig. 3b).

The average contents of saponins I and II were 30–40 and 10–15%, respectively using the isolated, pure compounds as standards.

It is known[7] that of 3-[2″-(β-D-glucopyranosido)-4′-(β-D-glucopyranosido)-β-D-

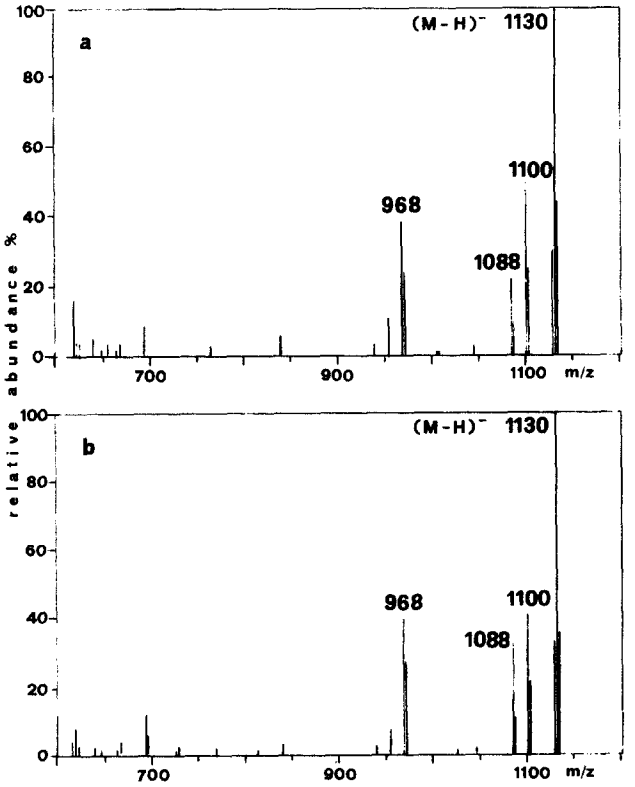

Fig. 3. Negative ion FAB mass spectra of saponin I (a) and saponin II (b).

glucuronopyranosido]-21-β-tigloyl-22-α-acetylprotoescigenin(I) and 3-[2″-(β-D-glucopyranosido)-4′-(β-D-glucopyranosido)-β-D-glucuronopyranosido]-21-β-angeloyl-22-α-acetylprotoescigenin the first is the major component. Therefore, on this basis and quantitation data, saponins I and II can be assigned as the tigloyl and angeloyl analogues, respectively.

In conclusion, the results of this study show the effectivenes of the method described for the separation of β-escin saponins. The isolation of the major constituents allows a new approach to the assay of β-escin in pharmaceutical and cosmetic formulations, and furthermore gives the possibility of a deeper insight into the pharmacological activity of each component.

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