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Note

Separation of major components in licorice using high-performance liquid chromatography

THOMAS H. BEASLEY, Sr., HOWARD W. ZIEGLER* and ALEXIS D. BELL

Corporate Analytical Services Laboratory, Mallinckrodt, Inc., P.O. Box 5439, St. Louis, Mo. 63147 (U.S.A.)

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Licorice extract (*Succus liquiritiae*) is the product of the aqueous extraction of licorice root, *Glycyrrhiza glabra*. The extract is an important commercial product used in the tobacco, food, and pharmaceutical industries throughout the world. Nieman[1] presented an interesting and informative treatise on licorice root, extract, chemistry, analysis, etc. The sweet tasting component of licorice extract is known as glycyrrhizin. The aqueous extract is a combined calcium–potassium salt form of glycyrrhizin acid, plus starches, gums, and various sugars. A pure form of glycyrrhizin is ammonium glycyrrhizinate.

A widely used method for the determination of glycyrrhizin is the acid precipitation procedure described by Houseman[2], which is slow and lacks specificity. Cundiff[3] presented a method where glycyrrhizin is assayed by hydrolysis to its aglycon, glycyrrhetinic acid, which is determined colorimetrically using the sulfuric acid–ethanol–vanillin reaction.

Methodology for the determination of glycyrrhizin has been reviewed by Steinegger and Marty[4], and by Zwaving[5]. Various methods were compared by Thieme and Hartman[6]. Thin-layer chromatography was used for the separation of glycyrrhizin with subsequent UV determination[7]. A gas–liquid chromatographic (GLC) method has been reported[8]. A GLC assay for ammonium glycyrrhizinate was presented by Larry *et al.*[9].

High-performance liquid chromatographic (HPLC) methods have been reported for the specific determination of glycyrrhizin[10,11]. These methods utilized anion exchange column packings with various mobile phases and buffers. Killachy *et al.*[12] report an HPLC determination for β-glycyrretinic acid only using a reversed-phase column packing after acid hydrolysis and chloroform extraction.

In this paper we describe a separation of at least eight major components in licorice extract using a gradient, reversed-phase HPLC system. Glycyrrhizin was identified at about 22 min based on the elution time of ammonium glycyrrhizinate.

* To whom correspondence should be addressed.

EXPERIMENTAL

Apparatus

The chromatograph used was a hybrid consisting of: a double beam UV detector (254 nm) with built-in 1-cm path length flow cell (Altex Model 151); a Milton-Roy minipump with pulse damper; a 10 mV full scale recorder; a Rheodyne Model 70-10, 10-μl sample injection valve; a stainless-steel column (500 × 3 mm I.D.) hand packed with Waters C_{18}–CORASIL (37–50 μm) (Waters Assoc., Milford, Mass., U.S.A.) reversed-phase packing; a stainless-steel precolumn (100 × 3 mm) containing Waters CORASIL II (37–50 μm) packing; and valves, tees, crosses, and connecting tubing to complete the system. The gradient elution system, consisting of a closed bulb, connected to a stream sampling valve (Altex), was an integral part of the chromatograph. A complete listing of the component parts, a schematic diagram, instrument operating parameters and construction of the closed gradient elution bulb were described in our publication on the assay of opium alkaloids using HPLC[13]. A modification of the chromatograph, which can also be used, was reported by us for the determination of gallotannin[14].

Reagents and solutions

The formic acid used for the system was distilled to remove UV absorbers. Pure distilled water, obtained from a Millipore (Bedford, Mass., U.S.A.) Milli-Q water purifier, was used. The initial mobile phase was 2% distilled formic acid in pure distilled water. The gradient program solvent was spectrophotometric grade acetonitrile. The ammonium hydroxide solution (4%) used for sample preparation was prepared by diluting 40 ml of reagent grade aqueous ammonium hydroxide to 1000 ml with pure distilled water.

Chromatographic conditions

Conditions used were as follows: flow-rate, 2 ml/min; pressure drop, 750 p.s.i.; detector, 0.32 a.u.f.s. at 254 nm; recorder, 10 mV; chart speed, 1 in./min; gradient bulb volume, 100 ml; solvent program, 0–60% acetonitrile; run time, 40 min.

Sample preparation and analysis

A 0.2 gram sample of licorice extract was dissolved in 100 ml of 4% ammonium hydroxide solution. A 10-μl volume of filtered extact solution was injected into a prepared chromatograph.

RESULTS AND DISCUSSION

At least eight components have been separated from licorice extract. Glycyrrhizin and glycyrrhetinic acid were positively identified by mixed pure component chromatograms and infrared determination of the peak fractions. Chromatograms of the same material were found to be reproducible from day to day.

Fig. 1a shows a chromatogram of purified, commercially available ammonium glycyrrhizinate. Fig. 1b shows the elution time of glycyrrhetinic acid (the aglycon). In Fig. 2, a trace of a typical licorice extract obtained from McAndrews & Forbes (New York, N.Y., U.S.A.) is displayed. A chromatogram

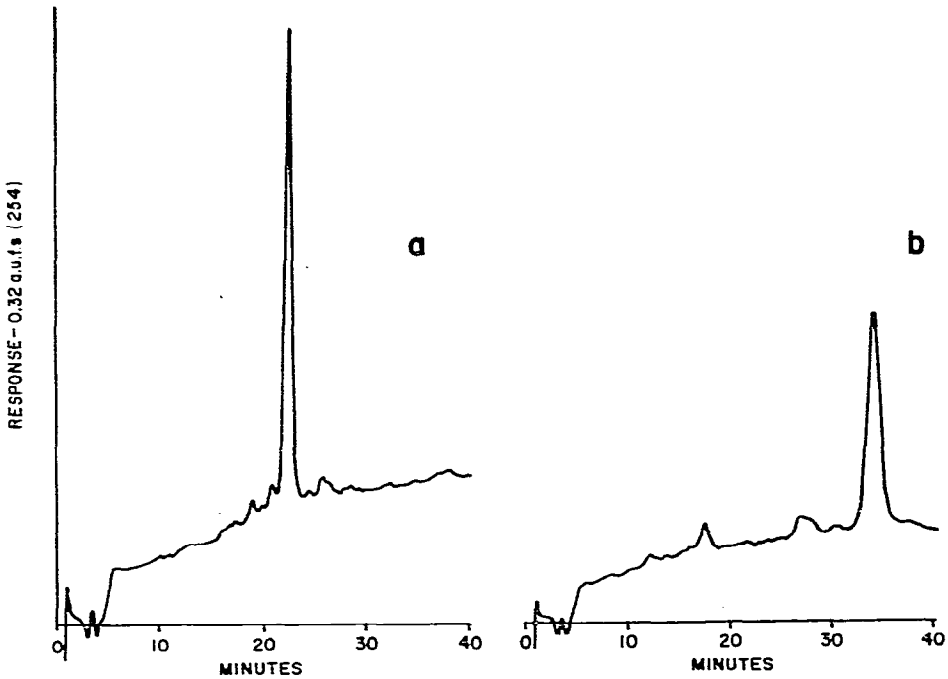

Fig. 1. Chromatogram of: (a) purified ammonium glycyrrhizinate; (b) glycyrrhetinic acid.

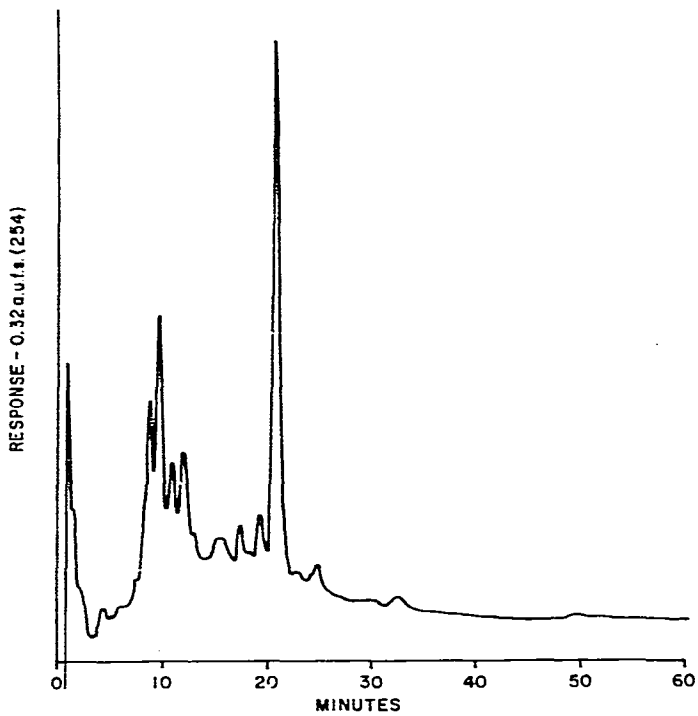

Fig. 2. Chromatogram of commercial licorice extract run for 60 min.

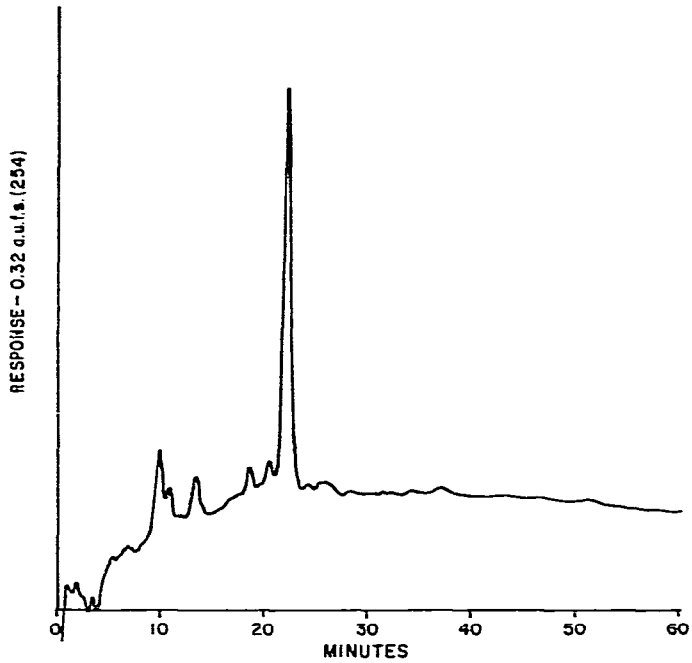

Fig. 3. Chromatogram of precipitate from the Houseman[2] assay of licorice extract.

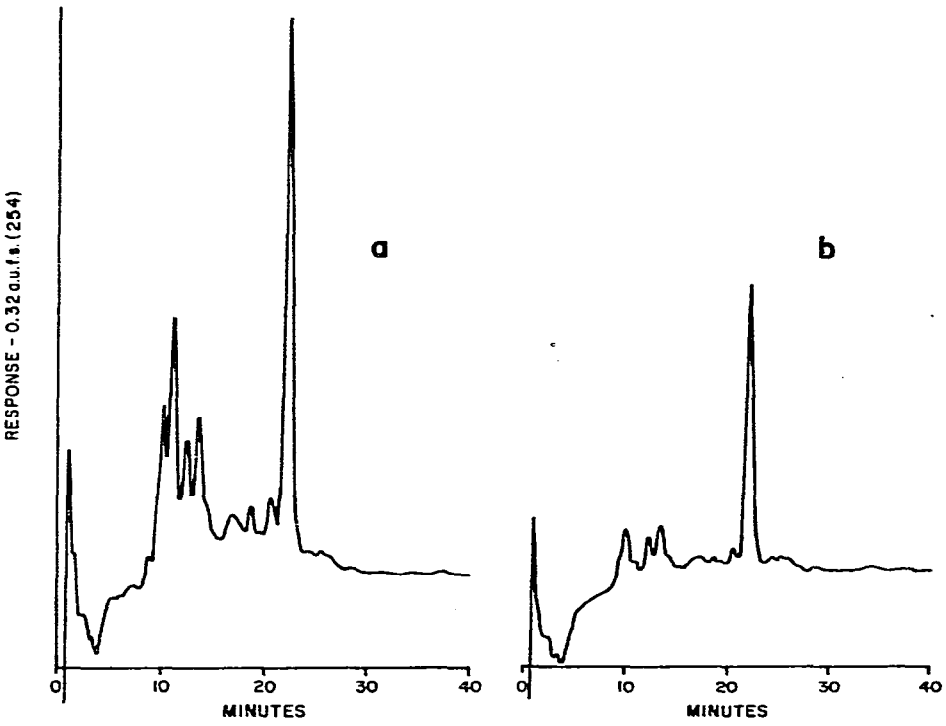

Fig. 4. Chromatograms of licorice extract originating from: (a) Israel; (b) Italy.

of a precipitate obtained from the Houseman[2] assay procedure is exhibited in Fig. 3. Curves from this assay appear to be very much like those of the licorice extract before the precipitation assay. We conclude that the Houseman method merely removes the alkali metals, starches, sugars, etc., but does not yield a purified glycyrrhizin. Fig. 4a is a chromatogram of an Israeli licorice extract, and an Italian extract is shown in Fig. 4b. Licorice extracted from roots obtained from Afghanistan is displayed in Fig. 5a, and a Russian extract appears in Fig. 5b.

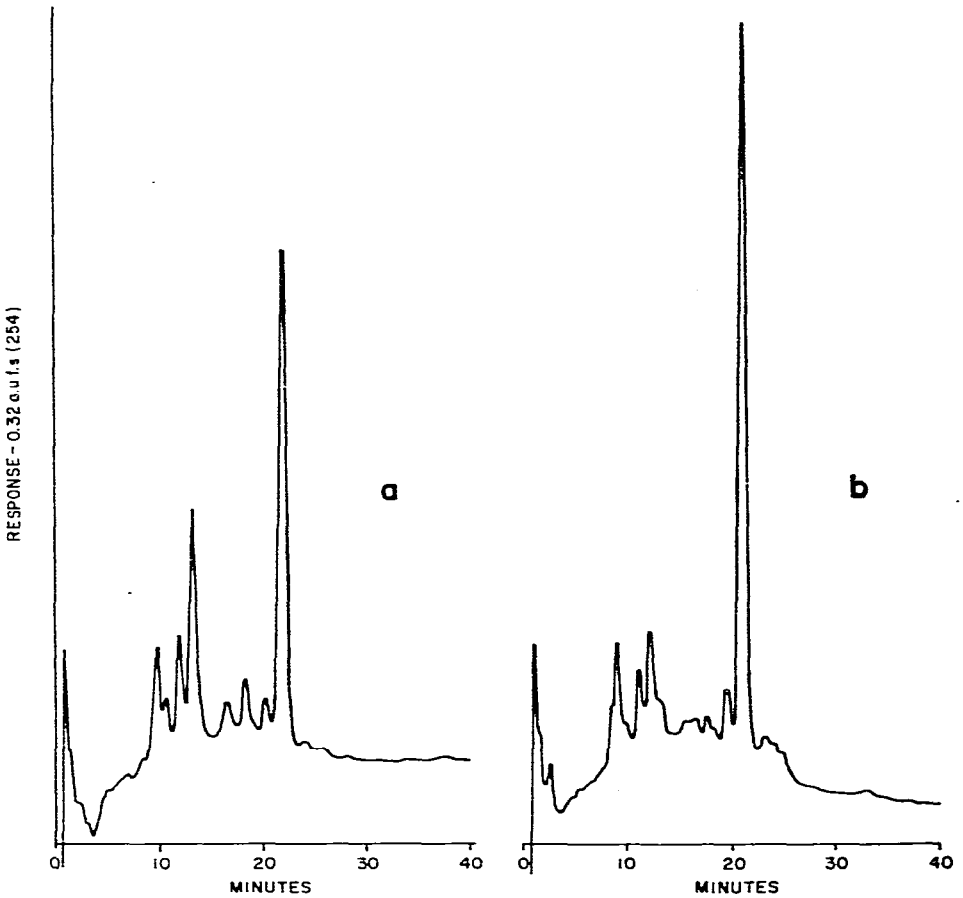

Fig. 5. Chromatogram of: (a) extract from Afghanistan licorice root; (b) Russian licorice extract.

The four peaks between 7 and 15 min elution time seem to vary in height depending on the country of origin. It is probable that the observed heights of the four peaks relative to each other are a "fingerprint" and could be used as a means of identifying the sources of root or extract. For instance, based on our chromatograms, it could be assumed that the extract from McAndrews & Forbes (Fig. 2) was extracted from Israeli licorice root (Fig. 4a). Other chromatograms we have prepared show different patterns for the four peaks with differing known countries of origin.

The HPLC separation presented here could be used for the assay of glycyrrhizin if a pure reference standard could be prepared. Ammonium glycyrrhizinate is a good candidate if the moisture and ammonium content is known.

An isocratic system, using the program solvent alone, gives reasonable separation but not as good as the gradient mode described here.

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