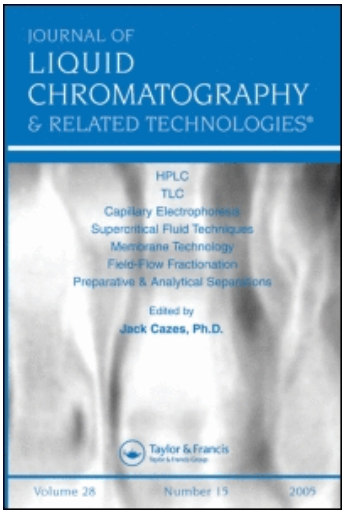

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ISOLATION OF GLYCOALKALOIDS WITH THE CHROMATOTRON* AND THEIR DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An extract of Solanum laciniatum leaves was fractionated with the Chromatotron. The glycoalkaloid fraction thus obtained was analyzed by reversed-phase HPLC. The load capacity of the Chromatotron with a 2-mm layer thickness is 600 mg (as determined with cholesterol) and the recovery of solasonine from the plant extract (as determined by HPLC) is 93-96%. The HPLC method permits the detection of as little as 1.5 µg solasonine or 3.5 µg solamargine with a linear detector response up to 375 µg for the former and 250 µg for the latter.

INTRODUCTION

In an earlier publication (1) we have described the analysis of the steroidal alkaloid in the fruits of Solanum khasianum by a combination of the Chromatofuge*** and HPLC. In plants, solasodine and other steroidal alkaloids occur in the form of glycosides. Two such glycoalkaloids occurring in S. laciniatum leaves are solasonine (solasodine + L-rhamnose

*Centrifugally accelerated radial thin-layer chromatograph manufactured by Harrison Research, 840 Moana Court, Palo Alto, CA 94306.

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+ D-glucose + D-galactose) and solamargine (solasodine + L-rhamnose + L-rhamnose + D-glucose). In connection with a project in Israel, we have developed a method for the isolation and determination of the solasodine glycosides.

For this work we have chosen to test the Chromatotron*, an instrument previously used by Hostettmann et al. (2) for the isolation of ginsenosides from ginseng. Quantitative analysis by HPLC has previously been applied to the solanidine glycosides by Bushway et al. (3-5). Our quantitative assay of the solasodine glycosides is based on a method by Crabbe and Fryer (6).

METHODS

Extraction

Freeze dried and powdered S. laciniatum leaves, weighing 22.2 g, were extracted for 24 hours in a Soxhlet extractor with 250 ml of 5% acetic acid in methanol. The extract was filtered and the residue on the filter paper was washed with methanol. The combined filtrates, which contained 6.87 g of dissolved solids, were diluted to 300 ml with methanol. A 100-ml aliquot of this extract was evaporated to a volume of less than 10 ml under reduced pressure, brought to pH 7.5 with conc. ammonium hydroxide, and filtered. The clear, dark-green solution was diluted to 25 ml with methanol-ethanol (9:1).

Chromatotron

The rotor of the Chromatotron is a frosted glass disk, ca 4 mm thick and 24 cm in diameter, with a 7-mm round hole in the center. The adsorbent layer, 2 mm in thickness, used for this work was prepared by slurrying a mixture of 50 g Silica Gel HF-254+366 Type 60-Merck (E.M. Laboratories, Scientific Products, McGaw Park, IL 60085) and 4 g $CaSO_4$ 1/2 H_2O in 50 ml of water. The slurry was added to the plate while the latter was spinning at 33 rpm on a converted record player. A polyethylene collar, made from a

conical wastebasket, prevented the slurry from running over the edge of the plate. The silica gel was then allowed to set for 5 hrs at room temperature and for 3 hrs at ca 80°C. The plate was scraped with the rotary scraping tool and finally with the finishing tool supplied by the manufacturer.

The thin-layer disk was mounted in the Chromatotron and, while it was spinning without the cover plate, a 5-ml aliquot of the extract (one-fifth of the sample) was applied, dropwise, to the inner edge of the adsorbent layer, followed by two 1-ml ethanol rinses. The cover plate was then installed and the chromatogram was developed with 250 ml ethanol at a rate of 2 ml/min, delivered by a single-piston reciprocating pump (Model 110, Altex-Beckman, Berkeley, CA 94710). The effluent from the Chromatotron was passed into a sump, from which it was pumped by another Altex Model 110 pump through a variable-wavelength detector (Model 155-10, Altex-Beckman), which was set at 210 nm and connected to an Omniscribe recorder (Houston Instrument, Austin, TX 78753), equipped with an event marker.

The sump was constructed from the barrel of a 3-ml hypodermic syringe with the hub pointing downward. The level of the liquid entering the barrel from the top was kept at 1 ml and controlled by means of a capacitance sensor (Thermocap relay, Niagara Electron Laboratories, Andover, NY 14806). The relay was connected to a clip, attached to the outside of the barrel at the desired level, and a grounded stainless-steel rod was placed inside the barrel. The relay activated the sump pump which was connected to the hub whenever the liquid level went past the top of the clip and shut it off when the liquid level fell below the clip. The effluent from the detector was collected in 10-ml portions in a fraction collector (LKB Model 7000, LKB Instruments, Rockville, MD 20852).

On the basis of our experiments with this system we devised the simple method of developing the chromatogram with 250 ml of ethanol. The eluate was collected and concentrated under reduced pressure to a volume of less

than 10 ml. A precipitate which formed was filtered off, and the filtrate and ethanol washes were made up to a volume of 10 ml with ethanol.

HPLC

The HPLC system consisted of an Altex Model 110 pump, connected through a sample injection valve with a 100- µl loop volume (Model 7125, Rheodyne, Cotati, CA 94928) to a Brownlee guard column (Rheodyne) and a prepacked stainless-steel column, 300 x 4.6 mm ID, both containing a 5- µm reversed-phase packing of silica gel-bonded octadecylsilane (IBM Instruments, Danbury, CT 06810). The detector was a Hitachi variable-wavelength spectrometer equipped with a flowcell having 10-mm pathlength and a 20- µl capacity, which was set at 210 nm and 0.1 AUFS. Signals from the detector were fed to a recorder, set at 10 mV.

The eluent was a mixture of acetonitrile:0.01 M Tris buffer (9:1), delivered to the column at a flowrate of 0.5 ml/min. Microgram quantities of solasonine and solamargine in methanol:ethanol (9:1) solution were injected and the detector response was determined. The quantitative assay was based on peak area measurements.

RESULTS AND DISCUSSION

Capacity and Efficiency of the Chromatotron

Most chromatographic analyses of natural extracts require a preparative "clean-up" step. The Chromatotron and the chromatofuge are both convenient instruments for purifying extracts either for isolation purposes or prior to quantitative analysis. In both instruments the solvent flow is accelerated by centrifugal force and the zones are sharpened during migration. However, they differ in load capacity.

The load capacity of the 2-mm layer of silica gel was determined with cholesterol as the sample and hexane:2-propanol (9:1) as the eluent. The maximum quantity of cholesterol giving a clearly defined zone without appreciable tailing was 600 mg. This is about the same as the load capacity determined for a 3-mm high layer of silica gel in the chromatofuge (1).

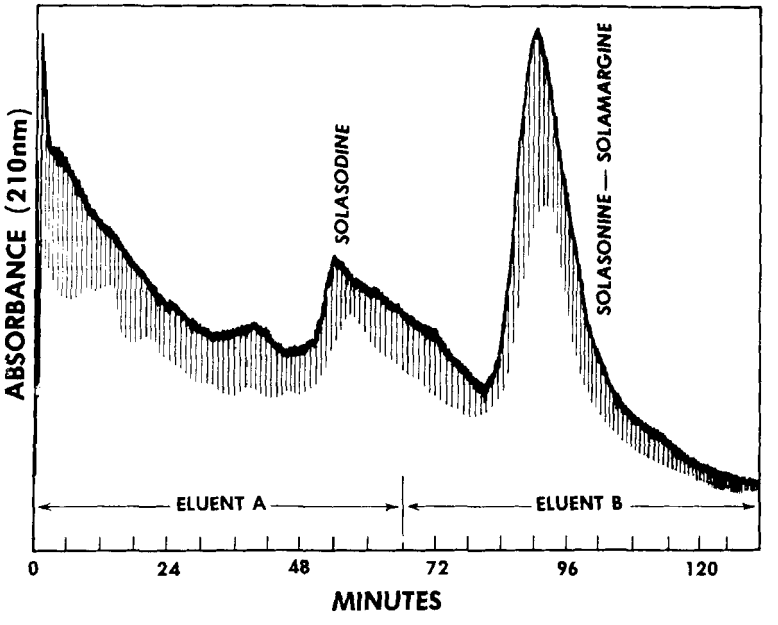

Fig. 1. Separation of solasodine from the glycoalkaloids, solasonine and solamargine, by the Chromatotron. A mixture of 7.9 mg solasonine, 1.4 mg solamargine, and 5 mg solasodine was applied to a 2-mm layer of silica gel. Eluent A, hexane:acetone:ethanol (18:1:1); Eluent B, ethanol. Coupling of the Chromatotron with a UV detector produced the tracing (see text).

Thus, the Chromatotron is equal to the chromatofuge in load capacity for approximately equal layer thickness. However, the layer thickness of the Chromatotron is limited to 4 mm by the cohesiveness of the binder, whereas the chromatofuge can be packed to 10 mm in height.

Fig. 1 illustrates the performance of the Chromatotron. It is a recorder tracing of an experiment in which 7.9 mg solasonine, 1.4 mg solamargine, and 5 mg solasodine were introduced into the Chromatotron, coupled with the UV detector. By elution with hexane:acetone:ethanol (18:1:1) for 66 min solasodine was recovered (peak at 54 min) and by elution

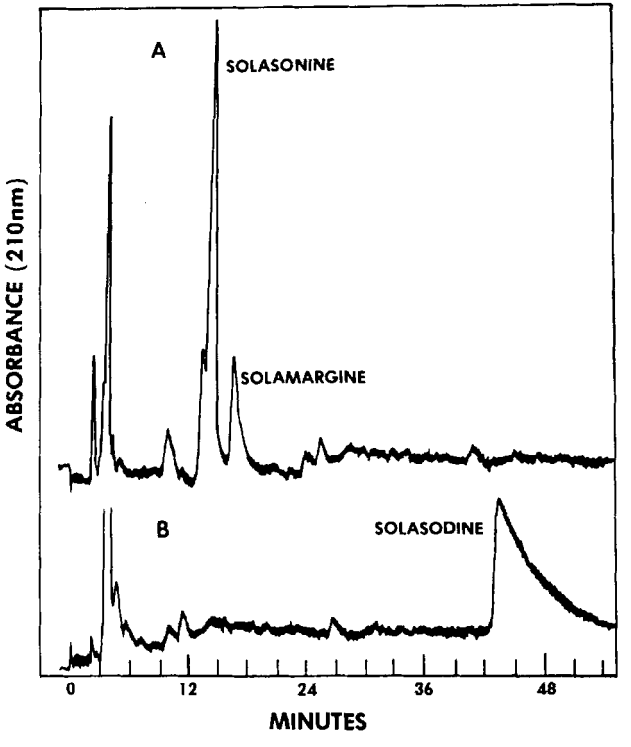

Fig. 2. HPLC of solasodine and its glycosides. Column, 300 x 4.6 mm ID, prepacked with silica ODS (5 μm); eluent, acetonitrile:0.01 M Tris (9:1); flowrate, 1 ml/min; pressure, 50 psi; UV detector at 210 nm, range 0.1; recorder speed 3 min/cm, span 10 mV.

A. Chromatogram of 10 μl S. laciniatum leaf extract (see text).

B. Chromatogram of the same extract after hydrolysis.

with ethanol solasonine and solamargine were obtained as a mixture (peak at 90 min). The vertical lines in the tracing are due to the intermittent operation of the pump delivering eluent to the detector.

Thus, while the glycoalkaloids were not separated from each other, they were clearly separated from the aglycone. An even simpler scheme suffices for separating the glycoalkaloids from miscellaneous contaminants in the plant extract (see Fig. 2A). Elution of the Chromatotron with ethanol furnishes material suitable for HPLC analysis in a shorter time than use of the chromatofuge (1).

Efficiency of HPLC

The acetonitrile:0.01 M Tris (9:1) eluent produces base-line separation of the two solasodine glycosides, solasonine (retention time 9.5 min) and solamargine (retention time 11.2 min) (6). Fig. 2 represents two chromatograms, produced under identical conditions. A illustrates the separation of the two glycoalkaloids in 10 µl (one-thousandth) of the eluate from the Chromatotron, while B shows the result of hydrolyzing an equivalent amount of the eluate as a test of identity.

Hydrolysis was carried out by a modification of the method of Crabbe and Fryer (7). The reaction was carried out in a 15-ml pear-shaped, stoppered flask, kept in a water-bath at 70°C for 3 hrs. A 200- µl aliquot of the eluate from the Chromatotron was treated with 3 ml of a mixture of methanol:water:conc. hydrochloric acid (80:9:7). After hydrolysis, the mixture was evaporated almost to dryness below 40°C under reduced pressure. The pH was adjusted to 7.5 with ammonium hydroxide and, after the addition of 400 µl dichloromethane with agitation, the dichloromethane layer was analyzed by HPLC.

Accuracy of the Chromatotron-HPLC Combination

Calibration curves for solasonine and solamargine were prepared by analyzing microgram quantities of the glycoalkaloids by HPLC. The plot of peak area vs. amount was linear for solasonine up to 375 µg and for solamargine

up to 250 µg. The slope of the calibration line (y = area in cm^2, x = amount in µg) for the former was 0.198 and for the latter it was 0.148. The minimum detectable quantity (signal/noise = 2) was 1.5 µg for solasonine and 3.5 µg for solamargine.

Recovery experiments were carried out by adding 5.1 mg, 5.6 mg, and 10.2 mg of pure solasonine to 5-ml aliquots of S. laciniatum extract whose solasonine and solamargine content had previously been determined and processing each sample as described under Methods. HPLC analysis of the Chromatotron eluates showed recoveries of 93%, 94%, and 96%, respectively, of the added solasonine. The experiments were repeated with the addition of 583 mg, 590 mg, and 594 mg of cholesterol with recoveries of 93%, 94%, and 95%, respectively, of the added cholesterol.

Solasonine and solamargine were identified in S. laciniatum by comparison of their retention times to known standards and by hydrolysis to solasodine. It was calculated that the dry leaves contained 2.2% of solasonine and 1.3% of solamargine. The combination of Chromatotron and HPLC thus permits the purification and analysis of these glkycoalkaloids in a natural extract.

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