Journal of Chromatography, 148 (1978) 521–527

CHROM. 10,425

Note

High-speed liquid chromatography of cardiac glycosides in milkweed plants and monarch butterflies

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(Received July 7th, 1977)

Cardiac glycosides (cardenolides) of milkweed (*Asclepiadaceae*) origin are chemicals of both toxicological and ecological importance. Compounds isolated so far have been found to differ structurally from their clinically useful counterparts such as digitoxin, digoxin, and ouabain. Most notable differences are in the stereochemistry of the A/B ring fusion, in the nature of the sugar molecules, and in the binding of the sugars of the A ring of the steroid nucleus[1] (see Fig. 1).

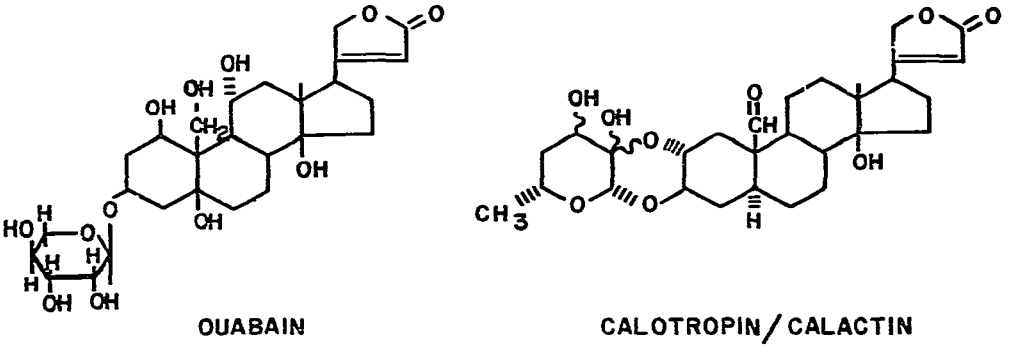


Fig. 1. Structures of Quabain and the *A. curassavica* milkweed cardenolide isomers, calactin and calotropin.

Insects of several orders sequester cardenolides from their milkweed food plants. The bitter nature and emetic properties of the stored compounds provide a means of protection for these insects against predators[2–4]. The monarch butterfly (*Danaus Plexippus*) represents one of the most documented examples of an insect that sequesters cardenolides. Monarchs feed on milkweeds during their larval stages and incorporate cardenolides within their tissues. The compounds are retained by the insect through metamorphosis and are stored in the adult butterfly. In laboratory studies, cardenolide-containing butterflies fed to environmental predator models (blue jays and starlings) cause them to vomit, indicating that the monarch may be protected from predators by the emetic response they elicit[1,5–8].

To understand better the chemical interrelationship between the monarch and

its milkweed host plant it is necessary to resolve and quantitate the individual cardenolides in extracts of plant and butterfly material. Thin-layer chromatography (TLC) coupled with specific detection of cardenolide spots using the chromogenic reagent, tetranitrodiphenyl (TNDP), is used in this laboratory for qualitative analysis of cardenolides in plant and butterfly material, but quantitation is difficult because the blue color of the cardenolide-TNDP complex is unstable and fades rapidly.

High-speed liquid chromatography (HSLC) has been applied to analysis of clinically important cardiac glycosides[9–15]. We report the use of reversed-phase HSLC for the quantitation of the major cardenolides in extracts of two milkweed species, *Asclepias eriocarpa*, and *A. curassavica*, and in extracts of monarchs whose larvae were reared on the species.

EXPERIMENTAL

Materials

A. eriocarpa plants were obtained near Woodland (Calif., U.S.A.). *A. eriocarpa* reared monarch larvae were captured in the field and rearing was completed to the adult stage in the laboratory. *A. curassavica* was grown in a greenhouse by Professor L. P. Brower of Amherst College. Butterflies from *A. curassavica* were reared completely in the laboratory at Amherst College. *Gonolobus rostratus*, a milkweed containing no cardenolides, and monarchs reared on it were analyzed as controls. Both *Gonolobus rostratus* plant and butterfly material were kindly provided by Professor Brower. Dried, powdered material was used in all cases. Plant and butterfly specimens from each species group were usually pooled for analysis.

Calotropin, calactin, and uscharidin were isolated from *A. curassavica* plants by a procedure employing solvent partition cleanup and column chromatography[16]. Identities were confirmed by comparison of melting points and infrared spectra with those reported by Brüschweiler *et al.*[17], and through TLC R_F values compared with authentic standards kindly furnished by Professor T. Reichstein. Labriformin, labriformidin, and eriocarpin were isolated from *A. eriocarpa* by a similar procedure. Their special properties suggest that they are related to the *A. curassavica* cardenolides, but their structures have not yet been assigned[18]. The R_F values of labriformin, labriformidin and eriocarpin with respect for digitoxin determined in two solvent systems are listed in Table I.

TABLE I

R_F DIGITOXIN OF *A. ERIOCARPA* CARDENOLIDES

Cardenolide	R_F *digitoxin*	
	Ethyl acetate–methanol (97:3) 2×	*Chloroform–methanol–formamide (90:6:1) 4×*
Labriformin	1.75	6.1
Labriformidin	2.2	6.1
Eriocarpin	1.7	2.7

Extraction and cleanup

Cardenolides were extracted from plant and butterfly material by incubating 0.1 gram of dried, powdered sample in 4.5 ml 95% ethanol for one hour at 70°–78° in a shaker water bath. Extracts were cooled to room temperature and the volume was brought to 5 ml with ethanol. 3 ml of the extracts were cleaned up for HSLC analysis using lead acetate for precipitation of pigments and fats. The procedure will be described in detail elsewhere. The volume of the cleaned extract was 3 ml. The total cardenolide concentration of the extracts were determined using the spectro-assay procedure developed by Brower and co-workers[19,20]. Extracts were concentrated for HSLC analysis.

High-speed liquid chromatography

The HSLC system consisted of a Milton Roy dual piston Mini Pump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.), a Rheodyne 20-μl fixed volume loop injector (Rheodyne, Berkeley, Calif., U.S.A.), and a Spectromonitor I UV–visible variable-wavelength detector (Laboratory Data Control). Separation was achieved using a 6.2 mm × 30 cm μBondapak C_{18} reversed-phase column (Waters Assoc., Milford, Mass., U.S.A.) eluted with a mobile phase of aqueous acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). The mobile phase was 25% acetonitrile for the assay of *A. eriocarpa* plant and butterfly material, while 30% acetonitrile was used for chromatography of *A. curassavica* samples. Wavelength of detection was 220 nm, the absorption maximum of cardiac glycosides due to the butenolide ring.

Chromatographic retention times of cardenolide standards were determined, and compared with peaks with similar retention times in extracts of plant and butterfly material. Identity of cardenolide peaks in sample extracts was confirmed by TLC. Standard curves were fit by linear regression analysis.

RESULTS AND DISCUSSION

Reversed-phase HSLC provided satisfactory resolution of the cardenolides of interest, slightly different proportions of mobile phase acetonitrile–water being optimum for the cardenolides of *A. curassavica* and *A. eriocarpa*. (Fig. 2.) Linear response was obtained to an approximate detection limit of 0.125 μg (per 20-μl injection at a detector sensitivity of 0.04 a.u.f.s.).

Eriocarpin, labriformidin, and labriformin were clearly visible in the chromatogram of *A. eriocarpa* plant extract (Fig. 3). The chromatogram of *G. rostratus* plant extract, run for comparison, suggests that labriformidin and eriocarpin peaks are superimposed on a sloping baseline contributed by non-cardenolide plant material, and that no non-cardenolide peaks are present that might represent serious interference for any of the three major cardenolides of *A. eriocarpa*.

Eriocarpin was the major cardenolide in the chromatogram of *A. eriocarpa*-reared butterflies (Fig. 4). Labriformidin and labriformin were not observed. The eriocarpin peak seems to be superimposed on a smaller, broader peak probably due to non-cardenolide material, as implicated by the peak in the chromatogram of *G. rostratus* butterfly extract at a similar retention time.

Analogous chromatograms of *A. curassavica* plant and butterfly extracts are

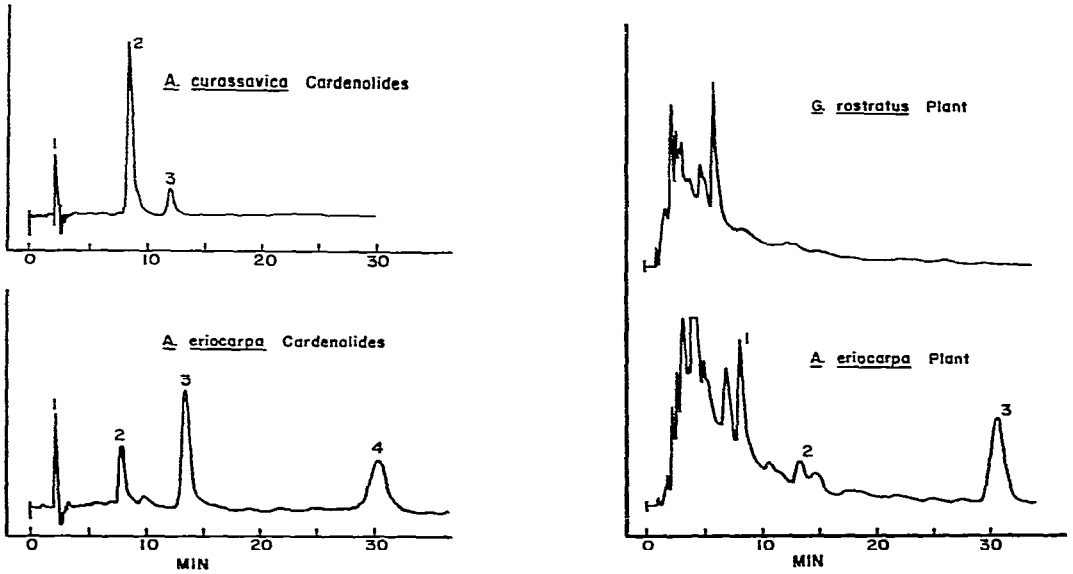


Fig. 2. Isocratic separation of cardenolides from the milkweeds *A. curassavica* and *A. eriocarpa* by HSLC. Conditions for *A. curassavica* cardenolides: μBondapak C_{18} column; mobile phase, acetonitrile–water (30:70); flow-rate 1.8 ml/min; UV monitor, 220 nm; attenuation, 0.08 a.u.f.s., temperature ambient. Peak identities: 1 = solvent; 2 = calotropin; (1.6 μg); 3 = calactin (0.4 μg). Conditions for *A. eriocarpa* cardenolides: Identical to those for *A. curassavica* cardenolides except that a mobile phase of acetonitrile–water (25:75) was used. Peak identities: 1 = solvent peak; 2 = eriocarpin (1 μg); 3 = labriformidin (2 μg); 4 = labriformin (1 μg).

Fig. 3. HSLC of *G. rostratus* and *A. eriocarpa* plant extracts. Conditions: μBondapak C_{18} column; mobile phase, acetonitrile–water (25:75); flow-rate 1.8 ml/min; UV detector, 220 nm; sensitivity, 0.08 a.u.f.s. Peak identities: 1 = eriocarpin; 2 = labriformidin; 3 = labriformin.

shown in Figs. 5 and 6. Once again, no major interferences due to non-cardenolide chemicals appear in the corresponding chromatograms of *G. rostratus*-derived samples.

Results of quantitative analysis of pooled and individual plant and butterfly samples are listed in Table II. Eriocarpin of *A. eriocarpa* and calotropin and calactin of *A. curassavica* are more concentrated in butterfly than in plant tissue. Labriformidin and labriformin, two major cardenolides of *A. eriocarpa*, are not sequestered by butterflies. In a similar way, uscharidin, a major cardenolide of *A. curassavica*, is not sequestered by butterflies reared as larvae on that plant, although we did not obtain quantitative values for it due to lack of a suitable standard. These results on sequestering are consistent with those made previously by TLC for *A. curassavica*[1] and for *A. eriocarpa*[21], particularly with regard to the selective uptake of a few plant cardenolides and the virtual exclusion of other, generally less polar cardenolides, by butterflies.

It should be noted that our analyses dealt only with the major cardenolides present in the two plants, and that several other such chemicals occur in each plant which could play a role in sequestering and antipredator defense. Other cardenolides present in the plants and butterflies have not been analyzed because they are present

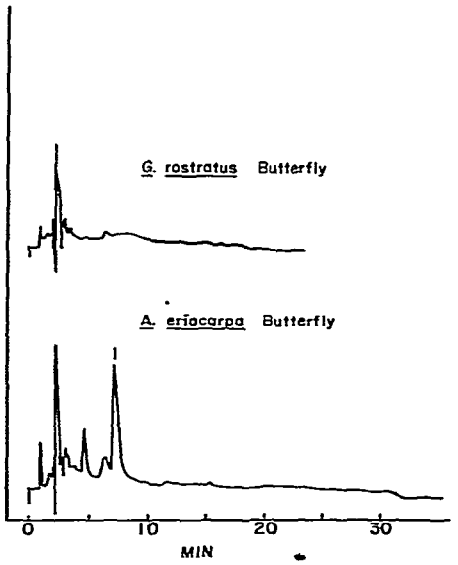
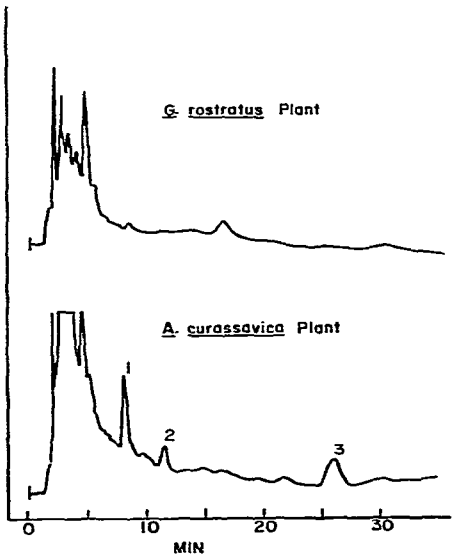


Fig. 4. HSLC of *G. rostratus* and *A. eriocarpa* butterfly extracts. Conditions identical to those in Fig 3. Peak identity: 1 = eriocarpin.



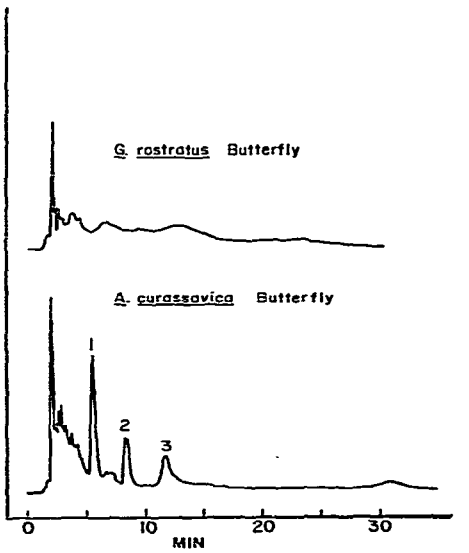


Fig. 5. HSLC of *G. rostratus* and *A. curassavica* plant extracts. Conditions as in Fig. 3 except mobile phase was acetonitrile–water (30:70). Peak identities: 1 = calotropin; 2 = calactin; 3 = uscharidin.

Fig. 6. HSLC of *G. rostratus* and *A. curassavica* butterfly extracts. Conditions as in Fig. 5. Peak identities: 1 = non-cardenolide material (as determined by TLC); 2 = calotropin; 3 = calactin.

TABLE II

AMOUNTS OF INDIVIDUAL MAJOR CARDENOLIDES IN MILKWEED AND MONARCH BUTTERFLIES

Source	*Cardenolide*	*Plant (μg/mg)*	*Butterfly (μg/mg)*
A. eriocarpa (pooled) *	Eriocarpin	1.07	2.06
	Labriformidin	0.21	0
	Labriformin	1.24	0
A. eriocarpa (sample no. 58) **	Eriocarpin	1.40	1.57
	Labriformidin	0.25	0
	Labriformin	0.71	0
A. eriocarpa (sample no. 282) **	Eriocarpin	0.64	1.20
	Labriformidin	0.13	0
	Labriformin	0.11	0
A. curassavica (pooled) *	Calotropin	0.41	0.62
	Calactin	0.15	0.41

* Result is an average of three determinations.
** Result is an average of two determinations.

in amounts below our limit of detection, because they chromatograph along with co-extracted non-cardenolide material which absorbs light at 220 nm, or because of lack of an appropriate cardenolide standard. Our present method has been applied successfully to analysis of the major cardenolides in a single butterfly and in as little as 0.1 g of dried plant, making quantitative ecological correlations of sequestering on an individual butterfly–leaf basis possible for the first time. The method may also be applicable to the analysis of cardenolides in other plant sources, and in other cardenolide-sequestering insects.

ACKNOWLEDGEMENTS

The authors are grateful to Professor T. Reichstein for providing standards of pure *A. curassavica* cardenolides and to Professor L. Brower for providing plant and butterfly material. The authors would like to thank Carolyn Roeske for her technical assistance and help in preparing this manuscript. Research supported in part by NIH ES00125 and NSF DEB 75-14266.

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