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Note

On-plate injection in the preparative separation of alkaloids and glycosides using overpressured layer chromatography

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Since overpressured layer chromatography (OPLC) was introduced by Tyihák and co-workers[1,2], several papers have appeared in the literature dealing with this technique. However, among the numerous analytical applications of OPLC, only a few descriptions of preparative work have been documented thus far. Recently, the use of OPLC as a new on-line preparative planar chromatographic method has been reported[3,4]. Using this procedure, no scraping and eluting of bands is necessary, since the components are drained from the plate and collected. We have recently shown the applicability of OPLC for the rapid preparative separation of natural products with antineoplastic activity[5].

In previous preparative OPLC methodology, the sample has been streaked onto the plate prior to development. This is often a tedious and time-consuming procedure. In this communication, we wish to report the use of on-plate injection as a rapid sample application technique for preparative OPLC. The utility of this method has been demonstrated for the purification of various plant alkaloid and glycoside constituents, comprising some canthin-6-one alkaloids from *Simaba multiflora* A. Juss, an iridoid glycoside from *Crossopteryx febrifuga* (Afzel ex G. Don) Benth., and two cardiac glycosides from *Streblus asper* Lour.

EXPERIMENTAL

General procedures

All separations were carried out with a Chrompres 25 OPLC system (Labor MIM, Budapest, Hungary). Plate-edge impregnation was performed with Impres II polymer suspension (Labor MIM). Precoated PSC-silica gel 60 F_{254} plates, 20 × 20 cm, 2 mm layer thickness (Merck, Darmstadt, F.R.G.) were used for all separations. The eluting solvent was delivered with an LDC/Milton Roy (Riviera Beach, Florida) mini pump VS, at a flow-rate of 3.0 ml/min. The cushion pressure during separation was kept at 20 bar. Prior to injection, the plates were conditioned with the appropriate solvent system for 30–45 min at a flow-rate of 4.0 ml/min. Sample injection was performed with a Multifit B-D syringe (2 ml). Fractions of 15–20 ml were collected in each separation. Purity of fractions was checked by thin-layer chromatography (TLC) using silica gel 60 F_{254} aluminum-backed plates (Merck). All solvents used were analytical grade.

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Samples

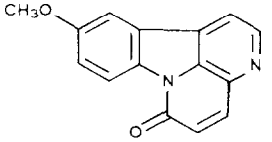
Crude 10-methoxycanthin-6-one (I), as well as purified 3-methoxycanthin-2,6-dione (II) and canthin-2,6-dione (III) were obtained from the wood of *Simaba multiflora* (Simaroubaceae)[6]. *Crossopteryx febrifuga* (Rubiaceae) stem bark, from which the iridoid glucoside, shanzhizide methyl ester (IV) was isolated, was collected in Ghana in June, 1977, and a voucher specimen representing this collection is deposited in the Herbarium of the National Arboretum, Washington, DC, U.S.A. A mixture of the cardiac glycosides mansonin (V) and strebloside (VI) was obtained from *Streblus asper* (Moraceae) stem bark[9].

RESULTS AND DISCUSSION

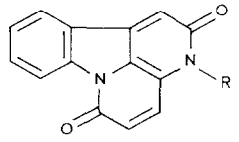
The following separations on the available alkaloids and glycosides were obtained in this investigation:

Purification of crude 10-methoxycanthin-6-one (I)

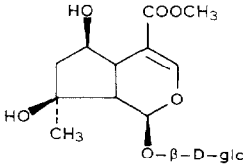
This sample was obtained from a gravity-fed silica column eluted with chloroform and increasing amounts of methanol[6]. TLC with ethylene chloride–tetra-



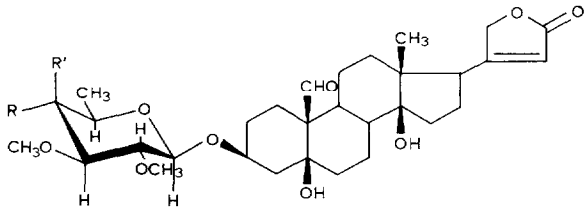
I



	R
II	OCH_3
III	H



IV



	R	R'
V	OH	H
VI	H	OH

hydrofuran (75:25) showed that the alkaloid was contaminated with two major impurities, possibly degradation products. The R_F values were 0.45 for the alkaloid and 0.41 and 0.35 for the impurities, respectively. Due to these close R_F values, a less polar solvent was chosen for the OPLC separation: hexane–ethylene chloride–tetrahydrofuran (20:60:20). A 44-mg sample was dissolved in 2 ml mobile phase, filtered, and injected onto the preconditioned plate. After 50 ml void volume, fractions were collected. Pure alkaloid eluted after about 40 min and was present in fractions 3–8 (24.8 mg). 10-Methoxycanthin-6-one (I) was previously shown to exhibit cytotoxic activity in the KB cell system[6].

Separation of 3-methoxycanthin-2,6-dione (II) and canthin-2,6-dione (III)

Compounds II and III were also obtained in our previous work on *Simaba multiflora*[6]. These compounds were well separated by TLC using chloroform–tetrahydrofuran–methanol (70:25:5) (R_F 0.62 and 0.34, respectively). The use of tetrahydrofuran increased the resolution between these two compounds. In the OPLC separation, 3 ml of mobile phase were added to a 1:1 mixture of these alkaloids (60 mg), and the mixture was sonicated in an ultrasound bath for 15 min, filtered, and injected onto the pre-equilibrated preparative plate. Due to the strong light-green fluorescence of these compounds in long-wavelength UV light, the chromatographic process could be observed through the methacrylate window of the Chrompres 25. After 20 min, fractions were collected. Compound II started to elute after about 30 min. After 60 min, the mobile phase was changed to chloroform–tetrahydrofuran–methanol (70:20:10), in order to accelerate elution of compound III. The separation was complete within 90 min. Fractions 1–5 and 11–14 contained, respectively, pure 3-methoxycanthin-2,6-dione (II, 27 mg) and pure canthin-2,6-dione (III, 22 mg).

Isolation of shanzhizide methyl ester (IV) from Crossopteryx febrifuga root bark

TLC analysis of the plant methanolic extract using the solvent system ethyl acetate–methanol–water (77:13;10) revealed the presence of a major component with R_F 0.20, which produced a violet-red spot with vanillin–sulfuric acid (1:99) spray reagent followed by heating. Using the above solvent system, isolation was attempted with preparative OPLC. The preparation of the sample was as follows: to 2.65 g crude methanolic extract, 10 ml mobile phase was added. The mixture was then placed in an ultrasound bath for 60 min to produce a latex-like insoluble residue and a dark brown supernatant. After preconditioning the plate, 6 ml of the supernatant were injected on the plate and the separation started. A total of 11 fractions was collected and the isolation was complete within 80 min. Fractions 6–9 contained pure isolate IV (42 mg).

The compound was then subjected to structural investigation. Interpretation of spectroscopic data, comprising UV, IR, electron impact mass spectrometry, two-dimensional ^1H–^1H homonuclear shift correlated (COSY) NMR, and ^{13}C-NMR, afforded the structure IV shown. Comparison with literature data showed that the isolated compound was identical with shanzhizide methyl ester, an iridoid glycoside which was earlier obtained from *Mussaenda parviflora* (Rubiaceae) by Takeda *et al.*.[7] It has not been found so far in the plant investigated herein. The compound was tested for cytotoxic activity using the KB cell system, according to establish protocols[8], but proved to be inactive.

Separation of the epimeric cardiac glycosides, mansonin (V) and strebloside (VI)

In previous work, a polar fraction from the stem bark of *Streblus asper* was found to exhibit cytotoxicity against the KB test system, and repetitive preparative TLC was necessary to isolate two cytotoxic components, mansonin (V) and its epimer, strebloside (VI)[9]. In the present study, a much more rapid and less laborious separation of these cardiac glycosides was achieved by preparative OPLC.

In the design of an OPLC system for the separation of V and VI, several different solvent systems were tried in TLC pre-assays, including various chloroform–methanol–water and ethyl acetate–methanol–water mixtures. However, the system which gave sufficient resolution on TLC was the upper phase of *n*-hexane–methyl *tert.*-butyl ether–*n*-propanol–ethanol–water (7:16:6:10:8) (mansonin, R_F 0.59; strebloside, R_F 0.41). This system was chosen for OPLC, and 90 mg of the fraction containing the glycosides was dissolved in 2 ml mobile phase. The plate was first prewashed with eluent, the sample injected, and the separation then started. Fractions 2 and 5–7 of the nine fractions collected contained mansonin (35.2 mg) and strebloside (26.5 mg), respectively. The total time for the separation of these pure isolates was only 60 min.

CONCLUSIONS

The feasibility of on-plate injection represents a further development in the use of preparative OPLC. Normally, OPLC separations are initiated on a dry plate in a non-equilibrated system. This very frequently leads to solvent demixing during the chromatographic run, causing ill-shaped bands ("zig-zag fronts"), which often results in loss of resolution. The phenomenon has been systematically studied by Wawrzynowicz and Soczewiński[10] for binary eluents in sandwich TLC chambers. However, it can be troublesome for the OPLC user.

If the sample is applied by on-plate injection, the chromatographic layer can be pre-equilibrated with the solvent system, thus avoiding the solvent demixing effect. Injection on a dry plate is not possible, because the sample would then migrate into the void area of the plate behind the inlet trough, and, in this way be excluded from the chromatographic development. However, once the plate is preconditioned, the solvent will only flow between the inlet and outlet troughs allowing effective injection.

All of the solvent systems employed in this work produce one or more fronts of higher order, which can be eliminated by pre-equilibration. In our experience, the stationary phase with a given layer thickness of 2 mm and plate dimension 20 × 20 cm is equilibrated within 30–45 min at a flow-rate of 4.0 ml/min, with most solvent systems.

A further advantage of on-plate injection is that the sample is applied very rapidly (in a few seconds) compared with the time-consuming streaking as is known for preparative TLC. In addition to this, the full separating range of the plate (18 cm) is available, if the sample is injected. It is also possible to perform up to two or three repeat injections on the same plate, depending on the complexity of the sample.

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