

CHROM. 16,853

Note

Reversed-phase ion-pair high-performance liquid chromatography of naphthazarins

STANLEY L. NICKEL*

College of Pharmacy, University of Toledo, Toledo, OH 43606 (U.S.A.)

and

THOMAS F. CARROLL

Toxicology Laboratory, Department of Pathology, Medical College of Ohio at Toledo, 3000 Arlington Avenue, Toledo, OH 43614 (U.S.A.)

(Received April 25th, 1984)

Naphthazarin pigments of the shikonin and alkannin series have a long history in the treatment of burns, skin diseases, and hemorrhoids in Japan and China¹. More recently, they have gained attention as potential anticancer^{2,3} and antibiomicrobial^{4,5} agents. Of particular importance to plant tissue culture research is the fact that *Lithospermum erythrorhizon* cultures can produce up to eight times more pigment than the original plant¹.

Two stereoisomeric series make up the naphthazarins as produced in plants. The shikonin series is of the *R*-form at the C-1 position of the six-carbon side chain and the alkannin series is of the *S*-form⁶. Figure 1 shows the structures of selected compounds in both series that have been isolated from plant material.

The first reported use of high-performance liquid chromatography (HPLC) for the study of shikonin pigments was reported by Fujita *et al.*⁷ who used a reversed-phase column and a solvent system consisting of acetonitrile-water-triethylamine-acetic acid (70:30:0.3:0.3). We attempted to use this solvent system with home-packed and commercial columns but were unsuccessful in obtaining any separation of the compounds. In this paper, development of an alternative HPLC method is described.

EXPERIMENTAL

Chromatographic procedures

A Varian 5000 HPLC equipped with a Vari-Chrom variable-wavelength detector operated at 520 nm was employed. A Varian 300 × 4 mm I.D. MicroPak Si-10 or a Varian 300 × 4 mm I.D. MicroPak MCH-10 column were used. The mobile phase for the normal-phase silica gel (Si-10) column consisted of hexane-ethyl acetate at variable ratios. For reversed-phase (MCH-10), the following mobile phases were used at variable ratios: solvent A: 0.01 *M* tetramethylammonium chloride (TMA) or nonylamine in acetonitrile-methanol (5:12); solvent B: 0.01 *M* TMA or nonylamine in water containing 0.01 *M* dibasic potassium phosphate adjusted to pH 3.0 with phosphoric acid. A flow-rate of 2 ml/min was used for all mobile phases.

Chemicals

Dibasic potassium phosphate, phosphoric acid and HPLC-grade acetonitrile, hexane, ethyl acetate, and methanol were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Nonylamine and TMA were obtained from Aldrich (Milwaukee, WI, U.S.A.). Authentic shikonin and β,β -dimethylacrylalkannin were kindly provided by Dr. M. Tabata (Kyoto University, Japan). The other alkannin standard test compounds: acetylalkannin, deoxyalkannin, β -hydroxyisovalerylalkannin and teracrylalkannin were isolated from *Macrotomia euchroma* plant material which was also provided by Dr. Tabata. Identification of each compound was confirmed by thin-layer chromatography (TLC) and combinations of nuclear magnetic resonance, mass spectrometry, infrared spectra, ultraviolet spectra and melting point.

RESULTS AND DISCUSSION

Development of an HPLC method for separation of the shikonin/alkannin compounds included two unsuccessful attempts in which the compounds were apparently retained on the column.

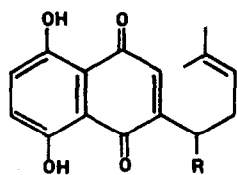
The first attempt consisted of the use of normal-phase MicroPak Si-10 silica gel column. This attempt was based on the successful separation of these compounds by silica gel 60 TLC utilizing a hexane-ethyl acetate (75:25) solvent system. The same ratio of solvents in the mobile phase of HPLC failed to elute any of the compounds tested. Varying the ratio from 100% hexane to 100% ethyl acetate did not improve the situation.

The second attempt consisted of the use of reversed-phase MicroPak MCH-10 column with various ratios of acetonitrile-water or methanol-water. Again, elution of the naphthazarins did not occur.

Use of ion-pair reagents was suggested in a paper by Gloor and Johnson⁸ for use in separating a variety of compounds. Because the naphthazarin structure (Fig. 1) lends itself to hydrogen bonding with each other and possibly to interactions with the absorbent as well, we felt that ion-pairing would effectively suppress this interaction. Basic ion-pairing reagents were chosen because the most likely interactions would be based on the acidic hydroquinone moiety of the molecules.

Two different basic ion-pair reagents were chosen. The first, nonylamine, caused elution to occur, but separation was poor for most compounds. For example, shikonin and β -hydroxyisovalerylshikonin eluted as one peak.

The second reagent, TMA, greatly improved separation of the six standard compounds and was used to produce the chromatograms shown in Figs. 2-3. Initially, TMA was used in methanol-water but tailing was severe, so acetonitrile was used to replace methanol. When this replacement was made the column became blocked whenever an injection was made, so the amount of acetonitrile was reduced until blockage no longer occurred. An optimum ratio of acetonitrile-methanol (5:12) was found that significantly reduced tailing and also did not cause blockage of the column. Various solvent A-solvent B ratios were used. A ratio of 85:15 provided sharp, symmetrical peaks but failed to separate three compounds: β,β -dimethylacryl, isovaleryl, and α -methyl-*n*-butyl derivatives of shikonin and alkannin. The ratio was adjusted down to 55:45, but elution time was unacceptably slow, so the temperature was raised to 70°C. At this ratio and temperature, complete separation of all of the



R	Compound	
	Alkannin series	Shikonin series
H	Deoxyalkannin	Deoxyshikonin
OH	Alkannin	Shikonin
OCO-CH ₃	Acetylalkannin	Acetylshikonin
OCO-CH=C(CH ₃) ₂	β,β -Dimethylacryl-alkannin	β,β -Dimethylacryl-shikonin
OCO-C(CH ₃) ₂	Not reported	Isobutylshikonin
$\begin{array}{c} \\ \text{H} \\ \text{OCO-CH}_2\text{C(CH}_3)_2 \end{array}$	Isovalerylalkannin	Isovalerylshikonin
$\begin{array}{c} \\ \text{H} \\ \text{OCO-CHCH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array}$	α -Methyl- <i>n</i> -butyl-alkannin	α -Methyl- <i>n</i> -butyl-shikonin
$\begin{array}{c} \\ \text{CH}_3 \\ \text{OCO-CH}_2\text{C(CH}_3)_2 \\ \\ \text{OH} \end{array}$	β -Hydroxyisovaleryl-alkannin	β -Hydroxyisovaleryl-shikonin
$\begin{array}{c} \\ \text{OH} \\ \text{OCO-CH}_2\text{CH=C(CH}_3)_2 \\ \\ \text{H} \end{array}$	Teracrylalkannin	Not reported

Fig. 1. Structures of naphthazarins of the alkannin/shikonin series.

earliest compounds was obtained. However, it still remained impossible to separate isovaleryl- and α -methyl-*n*-butyl derivatives.

To demonstrate the application of this method to actual plant extracts, we used benzene extracts of *Macrotomia euchroma* plant material (Fig. 2) and *Lithospermum erythrorhizon* tissue culture material (Fig. 3).

We found that the alkannin and shikonin series of compounds when co-chromatographed were indistinguishable, *i.e.* the *R* and *S* stereoisomers could not be resolved by this method.

The examination of the individual plant extract chromatograms revealed that the present HPLC method separated the deoxy, acetyl, β -hydroxyisovaleryl, and teracryl derivatives of alkannin and shikonin effectively. However, β,β -dimethylacryl derivative was only partly resolved from a mixture containing itself and isovaleryl- and α -methyl-*n*-butyl derivatives. Isovaleryl- and α -methyl-*n*-butyl derivatives eluted as one peak. All three of these compounds are known to be present in both *L. erythrorhizon* and *M. euchroma*. To date, the only method of identifying isovaleryl- and α -methyl-*n*-butyl derivatives is by gas-liquid chromatography-mass spectrometry of the hydrolyzed fatty acid side chains as described by Kyogoku *et al.*⁹. Neither of these constituents has yet been isolated as single, intact compounds.

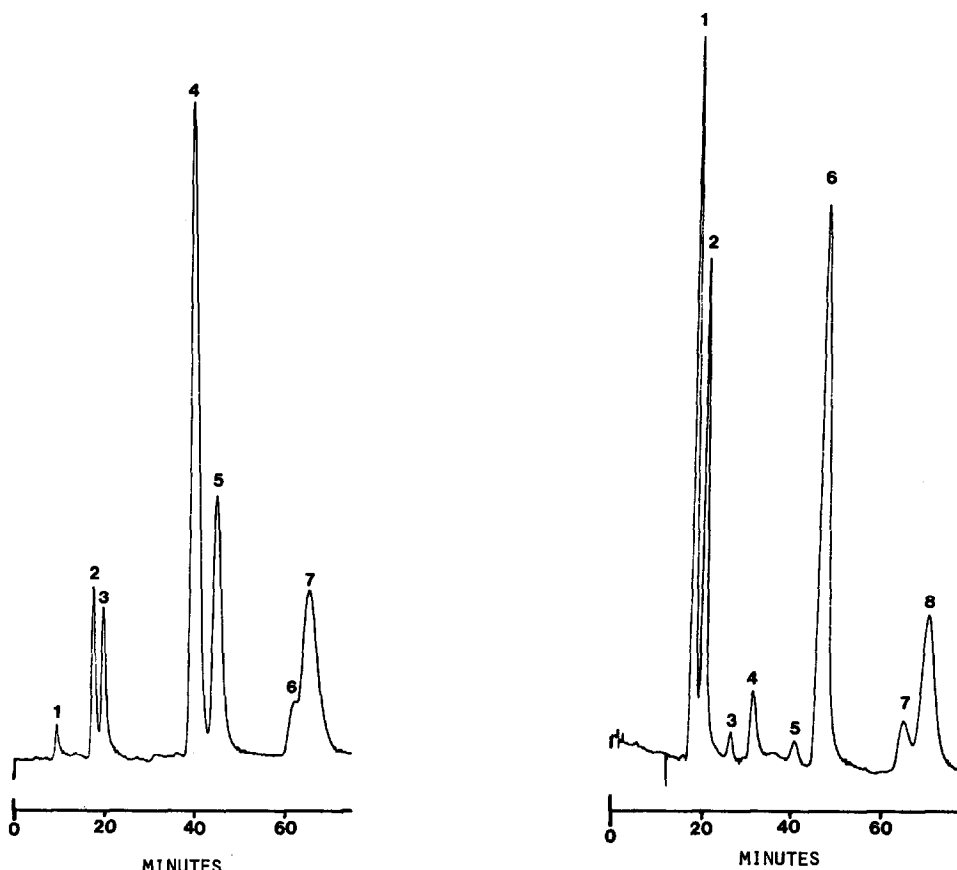


Fig. 2. HPLC profile of *Macrotomia euchroma* plant extract. Column: 300 × 4 mm I.D. filled with MicroPak MCH-10; mobile phase: solvent A-solvent B (55:45); temperature: 70°C; flow-rate: 2 ml/min; detector: 520 nm. Peaks: 1 = alkannin; 2 = β -hydroxyisovalerylalkannin; 3 = acetylalkannin; 4 = teracrylalkannin; 5 = deoxyalkannin; 6 = β,β -dimethylacrylalkannin; 7 = isovaleryl- and α -methyl-*n*-butylalkannin.

Fig. 3. HPLC profile of *Lithospermum erythrorhizon* tissue culture extract. Conditions as in Fig. 2. Peaks: 1 = β -hydroxyisovalerylshikonin; 2 = acetylshikonin; 3 = unknown, possibly echinofuran B¹⁰; 4 = unknown, possibly echinofuran C¹⁰; 5 = deoxyshikonin; 6 = isobutylshikonin; 7 = β,β -dimethylacrylshikonin; 8 = isovaleryl- and α -methyl-*n*-butylshikonin.

ACKNOWLEDGEMENTS

We express our gratitude to Dr. Mamoru Tabata, Kyoto University for reading this manuscript and for his gift of authentic shikonin and β,β -dimethylacrylalkannin standard compounds, and *Macrotomia euchroma* plant material. We also thank Dr. H. Fukui, Kyoto University for reading the manuscript. This work was supported in part by a grant from the Upjohn Company, Kalamazoo, MI, U.S.A.

REFERENCES

- 1 M. Tabata, in W. Barz, E. Reinhard and M. H. Zenk (Editors), *Tissue Culture and its Bio-technological Application*, Springer, New York, 1977, p. 4.
- 2 S. K. Gupta and I. S. Mathur, *Indian J. Cancer*, March (1972) 50.
- 3 U. Sankawa, Y. Ebizuka, T. Miyazaki, Y. Isomura, H. Otsuka, S. Shibata, M. Inomata and F. Fukuoka, *Chem. Pharm. Bull.*, 25 (1977) 2932.
- 4 M. Tabata, H. Mizukami, S. Naoe and M. Konoshima, *Yakugaku Zasshi*, 95 (1975) 1376.
- 5 V. P. Papageorgiou, A. Winkler, A. N. Sagredos and G. A. Digenis, *Planta Med.*, 35 (1979) 56.
- 6 H. Fukui, M. Tsukada, H. Mizukami and M. Tabata, *Phytochemistry*, 22 (1983) 453.
- 7 Y. Fujita, Y. Maeda, C. Suga and T. Morimoto, *Plant Cell Reports*, 2 (1983) 192.
- 8 R. Gloor and E. L. Johnson, *J. Chromatogr. Sci.*, 15 (1977) 413.
- 9 K. Kyogoku, H. Terayama, Y. Tachi, T. Suzuki and M. Komatsu, *Syoyakugaku Zasshi*, 37 (1973) 24.
- 10 H. Fukui, Kyoto University, personal communication.