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Note

High-performance liquid chromatographic determination of glaucine and its metabolite, dehydroglaucine in microbial culture

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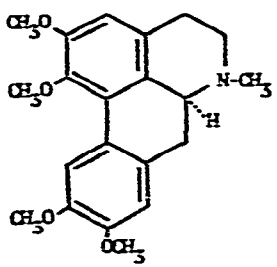
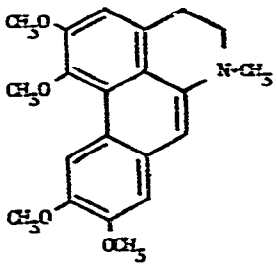
High-performance liquid chromatography (HPLC) has been utilized for the study of metabolic pathways permitting the separation and quantitation of residual starting material or substrate and metabolites or secondary products[1–3]. This application extends to areas including microbial transformations and microbial biosynthesis[4–6]. As with any analytical technique, rapid and accurate evaluation of the progress of metabolic events is highly desirable. Indeed, dramatic changes in metabolic profiles can take place over a short period of time, requiring a rapid judgement concerning changes in fermentation parameters to optimize yields[7,8].

We recently reported on the oxidation of glaucine (I) by *Fusarium solani* (ATCC 12823) to yield dehydroglaucine (II)[9]. Preliminary evidence indicated a relatively high yielding reaction exhibiting extensive stereoselectivity. Unfortunately the methodology utilized required isolation of both substrate and product by preparative thin-layer chromatography, followed by quantitation by UV spectroscopy and optical purity evaluation. Lengthy analytical methodology presents potential problems with dehydroaporphines because of their tendency to oxidize during isolation. Thus, an analytical method was desired which allows for rapid quantitation of glaucine and dehydroglaucine in microbial cultures for conducting quantitative stereospecificity studies. This was accomplished using the HPLC system described.

The devised method may also be useful in other natural products research,



I



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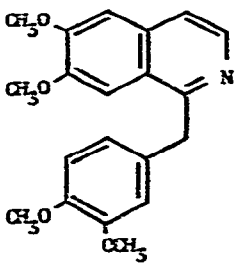

III

since aporphines and their respective dehydroaporphines are known to occur together in plant sources[10,11].

EXPERIMENTAL

Materials

Glaucine was synthesized by methylation of boldine with diazomethane by the method of Neumeyer *et al.*[12], and recrystallized from Skelly B. Dehydroglaucine was prepared by dehydrogenation of glaucine using 10% Pd/C as described previously[9]. Both standards yielded analytical data (m.p., nuclear magnetic resonance, infrared, mass spectra, optical rotation where applicable, chromatographic characteristics) identical to that reported previously[9]. Papaverine hydrochloride (Sigma, St. Louis, Mo., U.S.A.) gave analytical data consistent with the literature and was used as purchased.

Water used for HPLC was deionized and double-distilled in glass. Methanol and acetonitrile were HPLC grade (LiChrosolv®, MCB Reagents, Cincinnati, Ohio, U.S.A.). All other solvents and chemicals were of analytical reagent quality.

All glassware used in extractions was silylated using 2% trimethylsilyl chloride (TMSCl) (Pierce, Rockford, Ill., U.S.A.) in toluene, rinsed thoroughly and dried prior to use.

F. solani cultivation

Incubations to yield cells of *F. solani* (ATCC 12823, American Type Culture Collection, Rockville, Md., U.S.A.) were conducted essentially as described earlier[9], except a completely soluble growth medium was used consisting of dextrose (20 g), yeast extract (5 g, Difco Labs., Detroit, Mich., U.S.A.), sodium chloride (5 g), K_2HPO_4 (5 g), Acidicase Peptone (5 g, BBL Labs., Baltimore, Md., U.S.A.), water (1 l, distilled and deionized) and adjusted to pH 7.0 prior to inoculation. Cultures were harvested, combined, and fully homogenized (Polytron®, Brinkmann, Westbury, N.Y., U.S.A.) before use in these experiments.

Chromatographic procedure

A Tracor Model 950 pump and 970A variable-wavelength detector set at 280 nm were used. The unit was fitted with a Rheodyne Model 7125 injector with a 20-μl fixed volume loop (all injections were 20 μl). The column used was a μBondapak Phenyl® (Waters Assoc., Milford, Mass., U.S.A.), 10 μm, 30 cm × 3.9 mm I.D.

The mobile phase for isocratic elution was acetonitrile–methanol–0.05 *M* KH_2PO_4 (2:4:5). The mobile phase components were filtered separately (Whatman GF/F), then combined in the appropriate ratio, and degassed ultrasonically prior to use. The flow-rate used was 2.0 ml/min (1450 p.s.i.).

A Hewlett-Packard Model 3380A electronic integrator, programmed for analysis by peak area, was used for chromatographic recording and data generation. Statistical analysis of the resultant data was performed on a Wang Model 600 computer.

Extraction for analysis

Samples were analyzed using a single extraction step procedure. A solution consisting of 25 mg each of glaucine and dehydroglaucine in 2.0 ml methanol was prepared. Portions of 200, 160, 120, 80 and 40 μl were placed in duplicate in

125 × 16 mm silylated glass extraction tubes with PTFE-lined caps, and the solvent removed under a gentle nitrogen stream. Dimethylformamide (DMF) (10 μl) was then added (to be consistent with the mode of addition of the substrate to normal, growing cultures)[9]. A total of 5.0 ml of the *F. solani* culture homogenate and 500 μl of the internal standard solution (papaverine hydrochloride, 1.4 g/100 ml 0.2 *M* HCl) was added. The entire mixture was alkalinized with 1.0 ml 10% potassium hydroxide, and extracted with 2.0 ml ethyl acetate by gentle rocking (Lab-Tec aliquot mixer, 18 rpm) followed by centrifugation (1450 *g*, 2 min). A total of 50 μl of the organic phase was blown to dryness under a nitrogen stream, and reconstituted with 1.0 ml acetonitrile–methanol (1:1) prior to injection.

The resultant peak areas were used to plot standard curves for glaucine and dehydroglaucine as peak area ratios (standard/papaverine) *vs.* μg standard/ml culture. The glaucine curve typically yielded a slope = 0.002, *y*-intercept = 0.003 and $r = 0.9997$. The dehydroglaucine curve typically yielded a slope = 0.003, *y*-intercept = −0.472 and $r = 0.9990$.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatographic separation of glaucine (I), dehydroglaucine (II) and papaverine (III). Papaverine was chosen as an internal standard based on the achieved resolution and its ready availability. Other internal standards such as more polar aporphines were tried (*e.g.* boldine[13]) but it eluted with the solvent front. The wavelength chosen for detection (280 nm) was an optimal compromise for all three compounds.

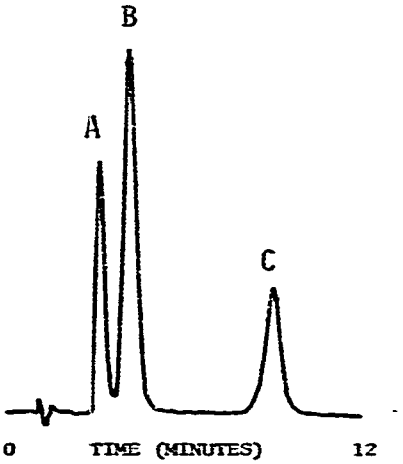

Fig. 1. HPLC separation of glaucine (A), papaverine (B) and dehydroglaucine (C). Conditions are described in the Experimental section.

The final composition of the mobile phase used in these experiments was a result of the systematic variation of the components of the ternary mixture until adequate resolution was obtained while retaining a short analysis time (10 min). A reasonably acidic system was desired to allow for complete ionization. The alternative,

development of totally unionized species, would require alkaline pH conditions which are known to contribute to column deterioration. The acetonitrile concentration was found to be particularly critical; at lower levels, poor resolution and "bullet-shaped" peaks resulted. This was the reason that the solvent-system components were filtered separately and then combined to generate the final mobile phase.

A relatively simple extraction procedure was devised that allows for the HPLC analysis of *F. solani* cultures after a single extraction step. Culture homogenates were spiked with levels of the standards glaucine and dehydroglaucine based on maximum theoretical substrate levels and product yield (500 μg/ml). Papaverine hydrochloride was added under strongly acidic conditions to allow complete solubility and permeability of the internal standard when added to growing cultures of *F. solani*. Following alkalinization and extraction, a clear supernatent resulted which was amenable to analysis. Only a small portion (2.5%) of the organic phase is taken and reconstituted for analysis. Obviously, if needed, much larger samples could be sampled and analyzed by the method. The resulting standard curves for glaucine and dehydroglaucine yield highly satisfactory results ($r = 0.9997$ and 0.9990 respectively) and demonstrates the utility of this analysis over the range anticipated for both compounds in microbial transformation experiments[9].

Attempts to apply a simplified procedure involving protein precipitation were unsuccessful[13]. Samples prepared as described above were precipitated under either acidic (pH 2) or alkaline (pH 9) conditions with three volumes of acetonitrile which yielded a clear supernatant following centrifugation. Both procedures yielded interferring peaks.

A method has been developed for the simple extraction and rapid analysis of glaucine and its metabolite. dehydroglaucine in *F. solani* cultures. This method has been applied to growing cultures of *F. solani* to follow the stereospecific oxidation of S(+), R(−), and RS-glaucine (results to be published elsewhere). This method may also have application in other areas of natural products chemistry as well. For example, aporphines and their dehydro-analogs often co-exist in plant extracts[10,11]. We are further examining the scope of this method for the analytical determination of other aporphines and dehydroaporphines, and the preparative scale separation of these compounds.

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