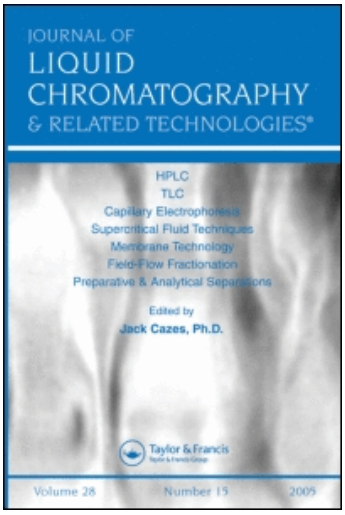

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Separation and Detection of Monopyrrole and Bipyrrole Precursors of Prodigiosin from *Serratia marcescens* by a Combined Method of High Performance Liquid Chromatography and Syntrophic Pigment Synthesis

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SEPARATION AND DETECTION OF MONOPYRROLE AND BIPYRROLE PRECURSORS OF PRODIGIOSIN FROM SERRATIA MARCESCENS BY A COMBINED METHOD OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SYNTROPHIC PIGMENT SYNTHESIS

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ABSTRACT

The monopyrrole (2-methyl-3-amylpyrrole) and bipyrrole (4-methoxy-2,2'-bipyrrole-5-carboxaldehyde) precursors of the tripyrrole pigment antibiotic prodigiosin, from Serratia marcescens were separated and detected by a combined method of high performance liquid chromatography (HPLC) and syntrophic pigment synthesis. The monopyrrole and bipyrrole precursors were extracted from mutants which were able to synthesize one but not both of the precursors. Mutant WF is incapable of synthesizing the bipyrrole while mutant 9-3-3 is unable to synthesize the monopyrrole precursor. By means of isocratic reversed-phase HPLC with a mobile phase of 70% methanol in water, the monopyrrole and bipyrrole precursors were well separated and resolved. The identity

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of the precursor peaks was estabilised by their ability to undergo syntrophic pigment synthesis with the corresponding cells. The syntrophically synthesized pigments shared similar spectroscopic as well as chromatographic characteristics as the parent tripyrrole pigment, prodigiosin, extracted from wild type of S. marcescens. The potential application of this method in the studies of prodigiosin biosynthesis and in the quantitation of monopyrrole compounds in clinical diseases is discussed.

Serratia marcescens produces a red tripyrrole pigment antibiotic, prodigiosin. A Bifurcated pathway involving the enzymatic coupling of the monopyrrole (2-methyl-3-amylpyrrole) (MAP) and the bipyrrole (4-methoxy-2,2'bipyrrole-5-carboxaldehyde) (MBC) intermediate precursors to form prodigiosin has been proposed by various workers (1,2,3). Most of the evidence for this pathway has been based on the syntrophic interactions of many mutants (2,3,4). Syntrophic synthesis is the cross-feeding of precursors for pigment formation in the cells. For example, mutant 9-3-3 which accumulates the bipyrrole precursor (MBC) or mutant WF which produces the monopyrrole (MAP) normally does not synthesize prodigiosin itself, but will produce prodigiosin if the missing precursor is present.

Although thin-layer chromatography and gas liquid chromatography have been used to separate and characterize MAP, high performance liquid chromatography (HPLC) has not been applied in the study of either the monopyrrole or the bipyrrole precursors of prodigiosin. The major advantages of HPLC over conventional chromatography are better resolution, shorter analysis time and superior precision, reproducibity and sensitivity. We have applied previously HPLC in the analysis of prodigiosin extracted from a wild type S. marcescens 08 (6,7). In this communication we report the results of HPLC separation of the monopyrrole and bipyrrole precursors. Instead of using the tedious and expensive chemical instrumentation for their identification, we used a combined method of HPLC retention time, reaction of Erlich reagent (p-N,N-'diemthylamino-benzaldehyde) and most importantly the ability to undergo syntrophic pigment synthesis for their detection.

MATERIALS AND METHODS

Chemicals. Pyrrole, 2,4-dimethyl-3-ethyl-pyrrole (kryptopyrrole) and pyrrole carboxaldehyde were purchased from Aldrich, Milwaukee, WI. All other chemicals used were of analytical grade. Solvents used for HPLC analysis were HPLC grade obtained from Fisher Scientific Co., Chicago, IL.

Growth of Bacteria

Serratia marcescens 08, a pigmented strain, was in stock in our laboratory. Mutants WF and 9-3-3 were kindly supplied by Professor R. P. Williams, Baylor College of Medicine, Houston, Texas. All cultures were grown in 2 l Erlenmeyer flasks containing 1 l of liquid medium containing 0.5% Bactopeptone and 1% glycerol on a rotary shaker at 27 C for 36-72 hr depending on the strain. Bacteria were harvested by centrifugation at 10,000 x g, and washed with distilled water. In the case of strain 08, harvested cells were submitted to pigment extraction according to the procedure of Williams et al. (8) with acetone followed by partition with petroleum ether. The supernatants of WF and 9-3-3 were saved for monopyrrole and bipyrrole precursors extractions.

Extraction of Monopyrrole and Bipyrrole Precursors

The supernatant of mutant WF after 36 hr of growth and the supernatnat of mutant 9-3-3 after 72 hr of growth were extracted with dichloromethane according to the modified method of Wasserman et al (9). The dichloromethane layers were collected after centrifugation at 10,000 xg for 25 min to break the emulsions. In the case of the WF the organic phase was washed twice with 1/10 volume of 1 N NaOH followed by washing with 1/10 volume of water. A liquid residue of monopyrroles (WF Extract) was obtained after the dried organic phase (with anhydrous Na_2SO_4) was removed by fractional distillation. The supernatant of mutant 9-3-3 was treated in a similar manner except that the organic phase was first washed with 1 N HCl followed by 1 N NaOH and then water. The dried organic phase was removed by distillation yielding the crude crystals of bipyrrole precursors (9-3-3 Extract).

HPLC Analysis

A Beckman model 330 isocratic High Performance Liquid Chromatography with a Pheodyne injector valve containing a 10 µl sample loop and a Perkin-Elmer

LC-55 variable wavelength UV-visible detector were used for separation and identification of the monopyrrole and bipyrrole precursors. The column was a 25 cm x 4.6 mm i.d stainless steel column and the precolumn was a 5 cm x 4.6 mm i.d. stainless column packed with Lichrosorb RP-18 of 10 µm particle diameter. The following solvent systems were developed to analyse the pigment and its monopyrrole and bipyrrole precursors from the various preparations:

a) 25% dichloroethylene in methanol with 10 ppm of conc. HCl was used for the separation of any formed pigments (6).

b) 70% methanol in water was used to separate both the monopyrrole and pyrrole precursors.

c) 50% acetonitrile in water was found to be an additional suitable solvent system for the separation of the bipyrrole precursors.

The flow rate was 2 ml/min for all solvent systems.

Syntrophic Pigment Synthesis

a) Syntrophic pigment formation in cells of 9-3-3 with monopyrrole precursor provided by cells of WF; Syntrophic pigment assays were carried out on Bactopeptone Glycerol-Agar agars. The upper half of each set of plates was heavily inoculated with 9-3-3 and the lower half was inoculated with WF. The plates were incubated for 36 hr at room temperature (22-24 C) and observed for pigment formation.

b) Syntrophic pigment formation in cells of 9-3-3 with monopyrrole precursor (MAP) or in cells of WF with bipyrrole precursor (MBC) separated and collected from HPLC analysis. WF Extract or 9-3-3 Extract from 2 l of supernatants of cultures was dissolved in 3 ml of dichloromethane for HPLC separation. The combined eluents (8-24 ml) after 5-6 injections of 10 µl each were extracted by adding 1 part of CH_2Cl_2 and 8 parts of H_2O. A small amount of the eluent before extraction was allowed to react with 0.5% p-N,N'-dimethylaminobenzaldehyde in ethanol/conc. HCl (Ehrlich-Reagent). The organic layers was evaporated to dryness in N_2. Two ml of the corresponding cultures of 9-3-3 or WF were added to the residue and incubated for 3 hr to allow pigments to form. Culture of 9-3-3 was added to peaks collected from WF Extract whereas culture of WF was added to peaks collected from 9-3-3 Extract.

Any pigment synthesized syntrophically was extracted by acetone as described for the pigment from cells of strain 08.

RESULTS AND DISCUSSIONS

Recently we have applied HPLC in the analysis of the pigment components of S. marcescens, a gram(-) bacterium frequently indicted for causing many nosocomial diseases. We have studied the conditions under which the pigment components could be separated (6.7). By maintaining a small but constant amount of concentrated HCl in the mobile phase of 25% dichloroethylene in methanol on a reversed phase column, the pigment components could be separated and resolved in about 5 minutes. This method allowed preparative isolation of the individual components along with their hydrogen peroxide oxidized products. We suggested that components separated by this method and detected in the ultraviolet region may represent the precursors of the parent pigment, prodigiosin. Indeed, MAP has been shown to be a chemical degradation product of prodigiosin (9) as well as a precursor for its biosynthesis (5). Systematic studies by HPLC of this and other degraded products may provide additional information in their possible role in the biosynthesis of prodigiosin.

Synthetic monopyrroles such as 2,4-dimethyl-3-ethylpyrrole (kryptopyrrole) having the structural similarity of MAP can also condense with MBC in a growing culture of 9-3-3 to produce the prodigiosin anologs. Other pyrrole derivatives such as pyrrole carboxaldehyde shares similar structural relationship with MBC. The UV-visible absorption spectra of these pyrrole derivatives provide us an important basis for the selection of the suitable wavelength for monitoring the analyses of the WF Extract and 9-3-3 Extract by HPLC (Figure 1). WF Extract had a maximum UV absorption at 210-235 nm which was shared by pyrrole and kryptopyrrole. 9-3-3 Extract had two maxima absorption (250 nm and 365 nm) in the UV spectra. The 250 nm absorption represents one of the two maxima of pyrrole carboxyaldehyde. An additional maximum absorption at 365 nm has also been reported for MBC.

Different results were obtained when the growth medium of mutant WF was extracted by various procedures under different conditions (Figure 2). Using

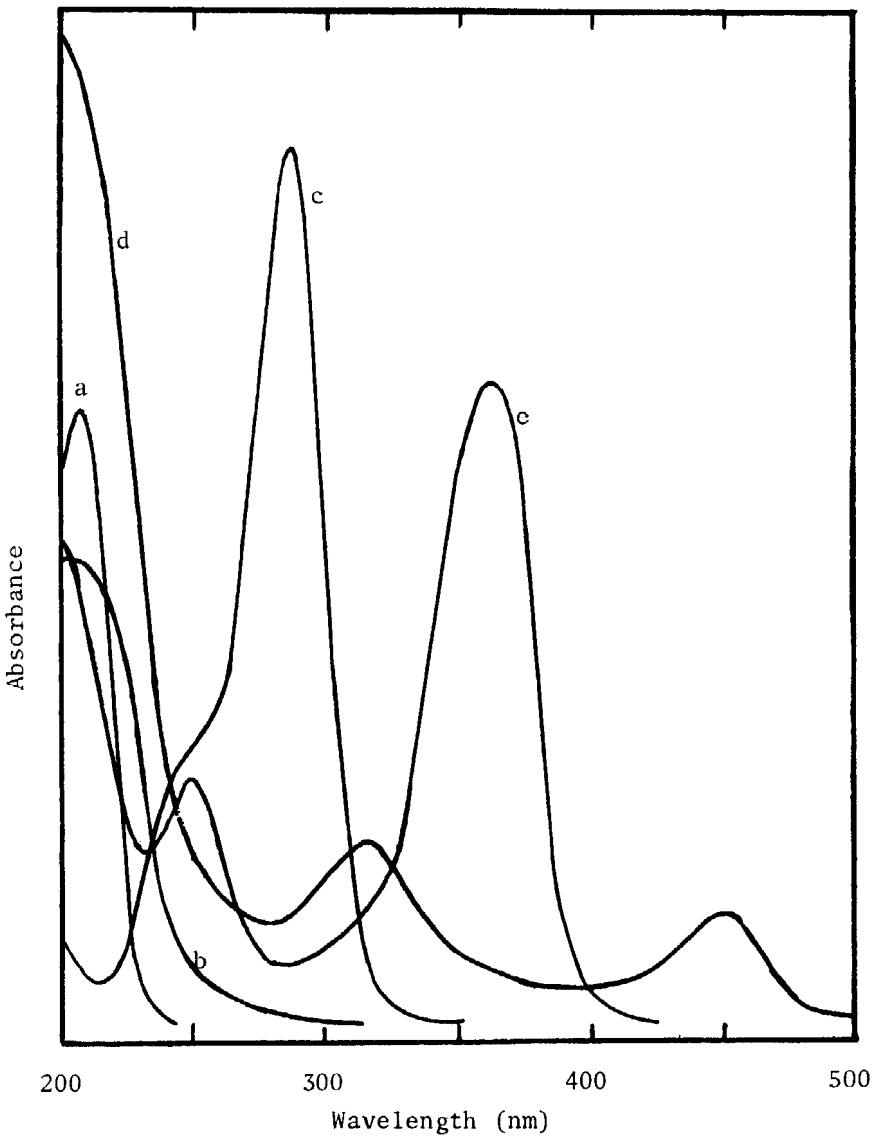

Figure 1. Ultraviolet-visible Spectra of pyrrole and pyrrole derivatives

a) Pyrrole

b) 2,4-dimethyl-3-ethyl-pyrrole (kryptopyrrole)

c) Pyrrole carboxaldehyde

d) Extract from mutant WF

e) Extract from mutant 9-3-3

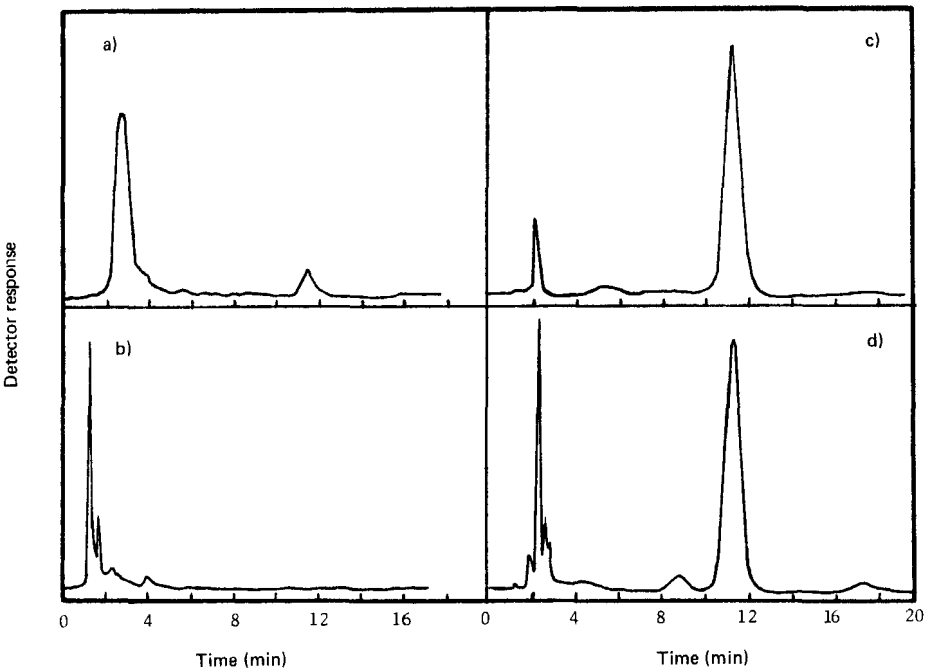

Figure 2. HPLC separation of monopyrrole precursors from S. marcescens mutant WF.

Column packing: Lichrosorb RP-18, 10 um particle size

Solvent system: 70% methanol in water

Detection: 223 nm at 20 mV, 10 mm/min

Flow rate: 2 ml/min

a) Fresh extract from growth medium with diethyl ether

b) Extract from growth medium with diethyl ether after two months storage in the cold.

c) Fresh extract from growth medium with diethyl ether followed by washing with 1 N NaOH

d) Fresh extract from growth medium with dichloromethane followed by washing with 1 N NaOH

70% methanol in water as a mobile phase, the chromatogram of the freshly prepared samples as extracted by diethylether according to the procedure of Deol et al. (5) showed two main peaks, a strong peak and a small peak (Figure 2-a). When this sample was stored in the cold for a period of two months, the first peak was degraded into several small peaks and peak 2 in the original sample almost disappeared (Figure 2-b). However, when either diethyl ether or dichloromethane was used as extracting solvent but washed with 1 N NaOH, four peaks with retention times of 2.3 min, 8.9 min, 11.4 min and 17.3 min were obtained (Figure 2-c and 2-d). The first peak (R_t = 2.3 min) had similar retention time and UV-visible absorption characteristics as pyrrole. Positive test with Ehrlich reagent (p-N,N'-dimethylaminobenzaldehyde in acidic ethanol) showed it was either pyrrole or pyrrole derivative. Peak 3 (R_t = 11.4 min) was the main peak with positive Ehrlich Reagent test and with absorption characteristics similar to those of kryptopyrrole (Figure 1). That this peak being a monopyrrole precursor such as MAP was confirmed by the syntrophic assay technique (Table 1). The remaining two peaks were not characterized, since neither one was present in any signficiant amount.

The chromatograms of the extracts from the growth media of mutant 9-3-3 (9-3-3 Extract) were fairly simple (Figure 3). When 70% methanol in water was used as a mobile phase, a single peak with retention time of 2.7 min was obtained when monitored at either 250 nm (Figure 3-a) of 365 nm (Figure 3-b). Again the synthrophic pigmentation test was positive when this peak was collected and allowed to react with the monopyrrole precursor, MAP, in the cells of mutant WF (Table 1). The UV-visible spectra were similar, if not identical, to those reported for MBC (5). Therefore, we conclude that this peak is most likely to be MBC. When 50% acetonitrile in water was used as the mobile phase, the retention time of this peak shifted slightly giving a retention time of 3.1 min (Table 1).

The results obtained in this study clearly indicated that peaks separated from either the extract of the supernatants of mutant WF or mutant 9-3-3 gave positive syntrophic pigmentation with cells of mutant 9-3-3 (test for MAP) or cells of mutant WF (test for MBC). The retention time and the absorption

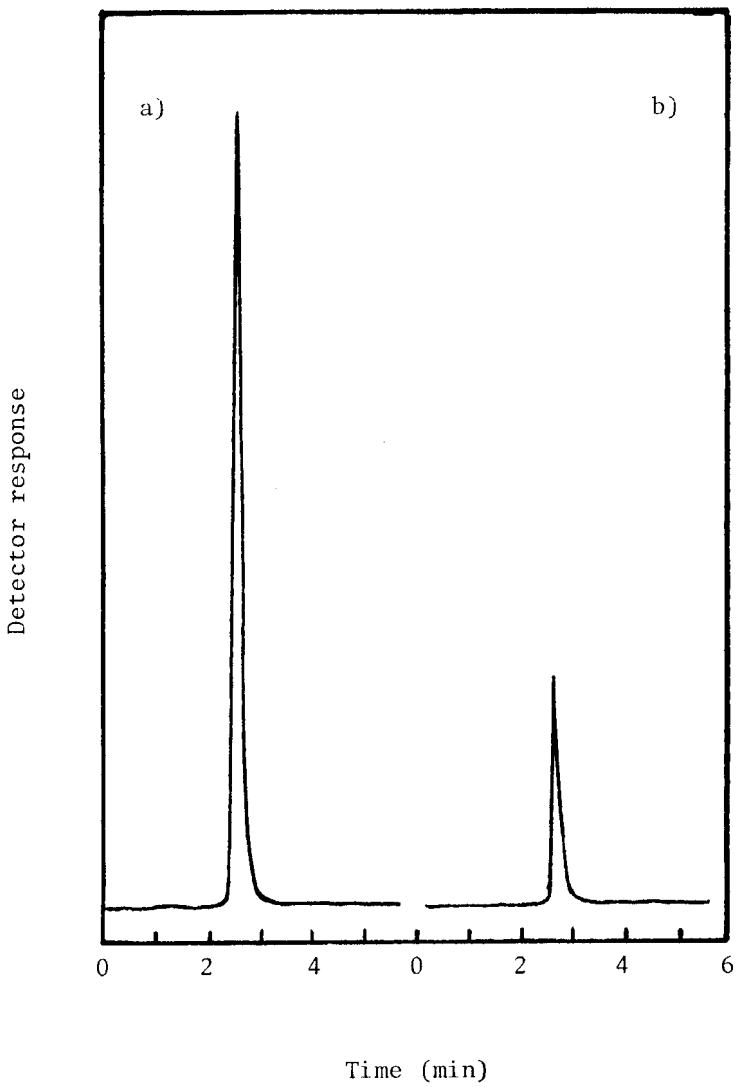

Figure 3. HPLC separation of bipyrrole precursor of S. marcescens mutant 9-3-3

Column packing and solvent system, see legend of Figure 2.

Detection: a) 365 nm, b) 250 nm

Table 1. Summary of Retention Time of Pyrrole and Pyrrole Derivatives in Various HPLC Solvent Systems and their Results with Ehrlich Reagent and In Syntrophic Pigmentation Synthesis

Compounds or Extracts	Detection Wavelength in nm	Retention Time in Various Solvents (min)			Ehrlich Test	Syntrophic Pigmentation Assay
		25% dichloro-ethylene in methanol	70% methanol in water	50% aceto-nitrile in water		
Pyrrole and Derivatives						
Pyrrole	223	1.8	2.2	2.8	+	-
2,4-dimethyl-3-ethyl-pyrrole	223	2.0	4.8	8.8	+	+
WF Extract from growth medium of WF (source of MAP)	223		2.2 8.9 11.4 17.3		+ - + -	- - + -
Pyrrole Carboxaldehyde	250	1.9	2.1	2.2	-	ND*
9-3-3 Extract from growth medium of 9-3-3 (source of MBC)	250 365		2.7 2.7	3.1 3.1	- -	+ +
Pigment Extracts						
Extract from cells of 08	537	7.7			ND	ND
Extract from 9-3-3 cells after reaction with MAP from WF cells	537	7.7			ND	ND
Extract from 9-3-3 cells after reaction with peak (R_t = 11.4) from WF Extract	537	7.7			ND	ND
Extract from WF cells after reaction with peak (R_t = 2.7) of 9-3-3 Extract	537	7.7			ND	ND

*ND = not done

spectra of the resulting syntrophic pigments were similar to those of the pigment of prodigiosin obtained from pigmented wild type strain 08 or the pigments obtained after syntrophic synthesis between cells of mutant WF and cells of mutant 9-3-3 (Table 1). These results provide strong but simple evidence for the presence of an active monopyrrole precursor in the growth medium of mutant WF (MAP) and an active bipyrrole precursor (MBC) in the growth medium of mutant 9-3-3. In order to study the separation and resolution of the monopyrrole and bipyrrole precursors in the sample, equal amount of WF Extract and 9-3-3 Extract was mixed and the mixture was analysed under the similar condition as described above (Figure 4-c). The bipyrrole (MBC) (R_t = 2.7 min) and the monopyrrole (MAP) (R_t = 11.4 min) were well separated and resolved. The reprodicibility of the separation provides for the first

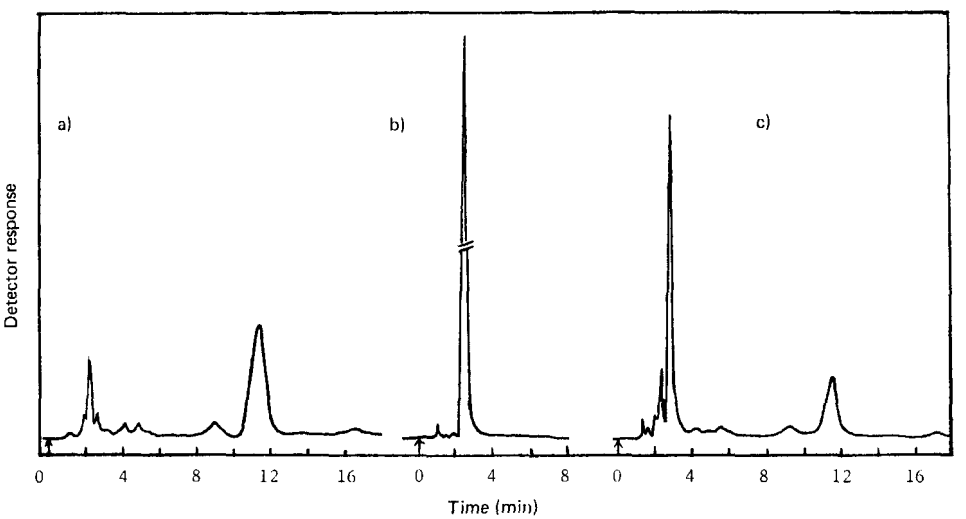

Figure 4. HPLC profiles of the mixture of WF Extract and 9-3-3 Extract
a) WF Extract b) 9-3-3 Extract c) mixture of WF Extract and 9-3-3 Extract (1:1, v/v)
Solvent system: 70% methanol in water
Detection: 235 nm

time a possible means to quantify the monopyrrole and bipyrrole precursors present in the same sample.

By combining the syntrophic pigmentation test and the powerful technique of separation and resolution of HPLC we believe we have established a simple and fairly rapid method for the detection of monopyrrole and bipyrrole precursors of prodigiosin. We are in the process of applying this method in the analysis of pigment and pigment precursors of various pigmented and non-pigmented clinical isolates of S. marcescens which might be responsible for many nosocomial diseases. Finally, it is worth mentioning that the method developed in this study could also be applied to the detection of other monopyrrole compounds in diseases, such as porphyria, psychosis and lead exposure (10).

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REFERENCES

1. Goldschmidt, M.C. and Williams, R.P. J. Bacteriol. 96, 609 (1968).
2. Morrison, D.A. J. Bacteriol. 91 1599 (1966).
3. Williams, R.P. and Hussain Qadri, S.M. The pigment of Serratia in Serratia Ed. A. von Gravaenitz and S.J. Rubin, CRC Press, Baca Raton, FL, p. 31, 1980.
4. Willams, R.P. and Green, J.A. J. Bacteriol. 72, 537 (1956).
5. Deol, B.S., Alden, J.R. and Still, J.L. Biochem. Biophys. Res. Comm. 47, 1378 (1972).
6. Kalanik, K., Webb, J.W. and Tsang, J.C. J. Liquid Chromatogr. 4, 61 (1981).
7. Feng, J.S. Webb, J.W. and Tsang. Appl. Environ. Microbiol. (in press).
8. Williams, R.P., Green, J.A. and Rappoport, D.A. J. Bacteriol. 71, 115 (1956).
9. Wasserman, H.H., McKeon, J.E., Smith, L.A. and Forgione, P. Tetrahedron, Suppl. 8, Part II, 647 (1966).
10. Moore, M.R. and Graham, D.J.M. Int. J. Biochem. 12, 827 (1980).