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High-performance liquid chromatographic analysis of *Pseudomonas aeruginosa* phenazines

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Abstract

An HPLC method is presented for the analysis of the major phenazine pigments of *Pseudomonas aeruginosa*: pyocyanin, 1-hydroxyphenazine (a product of degradation of pyocyanin) and phenazine-1-carboxylic acid. These compounds were determined in the supernatant of bacterial cultures with almost no need of any treatment. Samples were analyzed employing a C_{18} column varying the mixture of solvents from water–trifluoroacetic acid (100:0.04, v/v) to acetonitrile–water–trifluoroacetic acid (90:10:0.04, v/v/v) in several steps. The technique proved to be reproducible and highly sensitive. The calibration curves showed a linear response for ranges from 25 ng to 2.5 μg. Limits of quantification were: 2.1 ng for phenazine-1-carboxylic acid, 7.5 ng for 1-hydroxyphenazine and 13 ng for pyocyanin.

Keywords: *Pseudomonas aeruginosa*; Phenazines

1. Introduction

Different species of *Pseudomonas aeruginosa* share the ability to produce phenazine pigments throughout their secondary metabolism, when grown aerobically in phosphate-poor medium [1]. Although some precursors are common to the genera, each species presents its own pattern of pigments [2–10]. *P. aeruginosa* mainly produces two phenazines: pyocyanin (1-hydroxy-5-*N*-methylphenazine) and phenazine-1-carboxylic acid. The biosynthesis of these substances was studied by several authors and a pathway was proposed [11]. However the enzymes involved and their regulation are as yet unknown.

Interest in phenazines derives from its antibiotic properties, largely attributed to its redox cyclic nature [12,13] and from the correlation between its production and pathogenicity [14–18]. Moreover, we have found a great sensitivity *of P. aeruginosa* to ultraviolet-A [19] and ultraviolet-B [20] radiation and the role of phenazines as photosensitizers is currently under study at our laboratory.

The methods described in the literature for quantification of phenazines require extractions and subsequent TLC, scraping off the spot from TLC, elution and finally a colorimetric procedure. Such methods, albeit sensitive, seem relatively troublesome, taking into account the complexity of the samples, i.e. generally cultures or cellular pellets. A HPLC method was reported [21] for separating pyocyanin and 1-hydroxyphenazine, but the method requires extraction and does not resolve either phenazine-1-carboxylic acid or other phenazines.

In this paper, a modification of the HPLC method

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reported by Watson et al. [21] is described to measure pyocyanin, 1-hydroxyphenazine and phenazine-1-carboxylic acid from supernatants of *P. aeruginosa* cultures almost without any pretreatment of the sample.

2. Experimental

2.1. Bacterial strain and culture conditions

Pseudomonas aeruginosa ATCC 27853 wild type, pyocyanin$^+$, was used throughout. Bacterial cells were incubated at the indicated temperature with vigorous shaking, in the required medium.

The employed media were prepared as follows: (1) Luria-Bertani broth (LB): tryptone 10 g, yeast extract 5 g, NaCl 10 g, distilled water 1 l; (2) King A broth (KA) [22]: Bactopeptone 10 g, NaCl 5 g, glycerol 10 ml, K_2SO_4 1.4 g, distilled water 1 l; (3) modified M9 synthetic medium (MM): NH_4Cl 1 g, NaCl 5.5 g, K_2SO_4 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, K_2HPO_4 9.6 mg, disodium succinate hexahydrate 5.4 g, distilled water 1 l.

2.2. Bacterial phenazine preparations

Pyocyanin and phenazine-1-carboxylic acid were isolated from an overnight culture of *P. aeruginosa* ATCC 27853 grown in KA employing the isolation method previously described by Chang and Blackwood [23].

1-Hydroxyphenazine was obtained by pyocyanin degradation in alkaline solution as described by MacDonald [24]. The product was resublimed twice.

2.3. Identification techniques

Melting points were determined in kofler.

Ultraviolet and visible absorption spectra were obtained with a GBC UV–visible 920 spectrophotometer, GBC Scientific Equipment, PTY, Dandenong, Australia.

2.4. Preparation of samples for HPLC analysis

A culture (5 ml) was centrifuged for 10 min at 10 000 *g* at room temperature and the supernatant was acidified with approximately 30 μl of a water–trifluoroacetic acid solution (50:50, v/v) to pH 4. After 1 h, this liquid was centrifuged at the conditions described above and the supernatant was separated. A sample up to 1 ml was filtered by regenerated cellulose membrane filter (Schleicher and Schuell, reference number 410301, RC 58, pore 0.2 μm, diameter 13 mm) and injected into the HPLC.

2.5. Phenazine determination by HPLC

To determine the presence and concentration of phenazines, samples were analyzed according to a modification of the methods previously described by Watson et al. [21]. A 150×4.6 mm Ultracarb 5 μm ODS (30) column and a 30×4.6 mm Ultracarb 5 μm ODS (30) guard column were used. Solvent A was water–trifluoroacetic acid (100:0.04, v/v) and solvent B was acetonitrile–water–trifluoroacetic acid (90:10:0.04, v/v/v). Chromatography was developed using a Beckman System Gold programmable solvent module 126 connected to a Beckman diode array detector module 168. Elution was as follows:

Solvent A was maintained for 15 min and then changed to 90% A and 10% B. This mixture was used for 10 min. A linear gradient to 70% A and 30% B in 15 min was then applied. These conditions were maintained for 5 min (until 45 min from onset). Finally, solvent composition was changed to 64% A and 36% B and maintained until the end (65 min from the starting time). Running was monitored at 250, 262 and 280 nm with a bandwidth of 4 nm in all cases. When complex culture media were used, 390 nm instead 280 nm was employed. Whole spectra (between 190 nm and 598 nm) were recorded during elution of each peak.

2.6. Recovery determination

Recoveries were tested by adding standards to a complex sample at two concentrations: 1.0 μg ml^{-1} and 12.5 μg ml^{-1} for each compound. The average of areas were used to calculate the added standard concentrations, taking into account the regression results of both standard calibration curves and standard added calibration curves. The estimated con-

centrations were related with the real added concentrations to obtain recoveries as percents.

3. Results and discussion

3.1. Purification and identification of standards

Phenazine pigments of *Pseudomonas* have been studied by several authors [1–13,25] who have developed methods for isolating and purifying this type of substance. We have extracted and purified phenazines from cultures of *P. aeruginosa* employing these well known methods. Thus, we can be practically sure about the quality and identity of the isolated phenazines. Despite this, we checked some physical characteristics of our standards (Table 1) and we found them to be identical to those previously described [23–25]. Furthermore, HPLC analysis of a mixture of our standards showed no other peaks than those corresponding to the expected peaks (Fig. 1).

3.2. Selection of detector conditions and chromatography of standards

Standards were separately run on HPLC (see Section 2), to obtain the spectra of the phenazines under our running conditions (Fig. 2). The highest sensitivity was obtained choosing the wavelength at maximum absorbance for each pigment. The maximum absorptions were at 250, 262 and 280 nm for phenazine-1-carboxylic acid, 1-hydroxyphenazine and pyocyanin, respectively. These wavelengths were used to monitor the chromatography and peak areas were used in the calibration curves of the respective substances. However, since samples obtained from rich culture media (LB and KA) showed some interference at the pyocyanin peak, we used 390 nm instead of 280 nm in those cases.

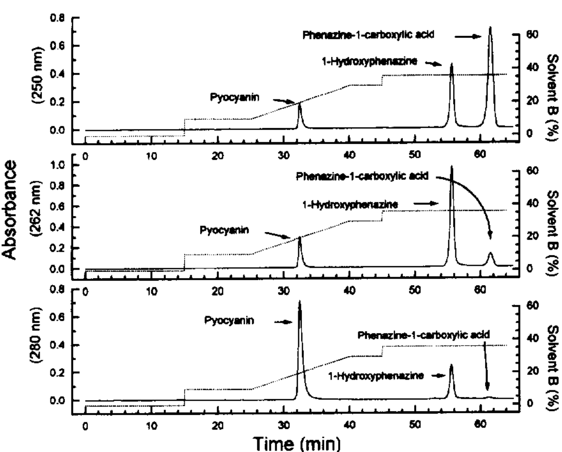

Fig. 1. Chromatograms resulting from the HPLC analysis of the standard mixture of the three studied phenazines. Concentrations were 25 $\mu g\ ml^{-1}$ and 100 μl were injected. Wavelengths employed for monitoring are indicated in parentheses at ordinate axes.

After optimizing the chromatographic conditions (see below) the peaks of standards pyocyanin, 1-

Table 1
Properties of crystalline phenazine standards

	Phenazine-1-carboxylic acid	1-Hydroxyphenazine	Pyocyanin
Appearance	Yellow fine needles	Yellow fine needles	Dark blue needles
Melting point (uncorrected) (°C)	237–240	157–158	132–134
Absorption maxima (nm)			
in Cl_3CH	369, 251	368, 360, 350, 264	690[a], 327, 309, 242
in 0.2 *M* HCl	370, 250, 211	383, 364, 274, 242	520[a], 388, 278, 207
in methanol	–	368, 360, 350, 263, 238	690[a], 368, 318, 238
Chemical structure	COOH, N, N	OH, N, N	OH, N, ⊕N, CH_3

[a] Broad absorbance.

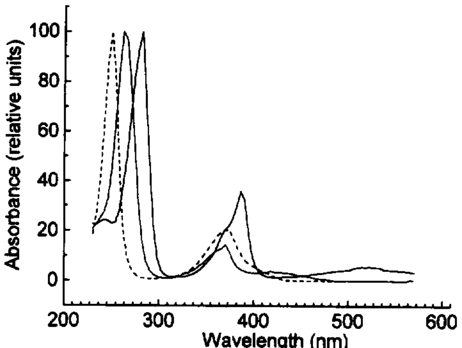

Fig. 2. Comparison of phenazine spectra in the running conditions: (- - -) phenazine-1-carboxylic acid, (· · ·) 1-hydroxyphenazine, (——) pyocyanin.

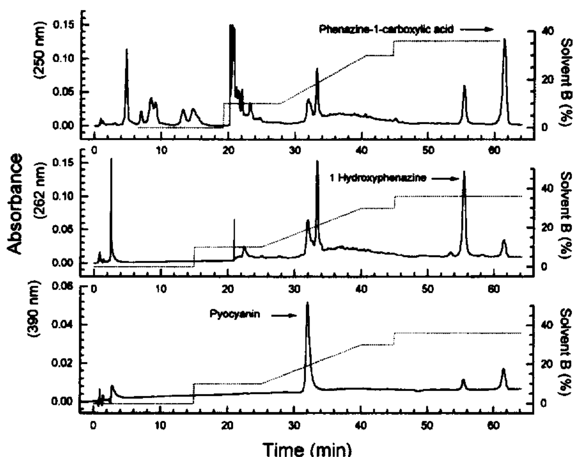

Fig. 3. HPLC analysis of supernatant of *P. aeruginosa* cultured in a complex medium (KA). Wavelengths employed for monitoring are indicated in parentheses at ordinate axes. Samples of 50 μl were injected.

hydroxyphenazine and phenazine-1-carboxylic acid were completely separated with retention times of 32.4, 55.8 and 60.5 min respectively as shown in Fig. 1.

3.3. Chromatography of samples

We formerly used an Hypersyl 5 μm ODS column (100 mm×4.6 mm OD) but pyocyanin showed an extensive tailing effect, possibly due to the quaternary nature of this phenazine. Therefore, this compound could interact through the positive charge of the phenazinium with the residual silanol groups of the support of the stationary phase. Thus we decided to try an Ultracarb ODS (30) column (see Section 2), since it has the highest carbon load and it was designed for use with polar compounds such as basic drugs (as insured by the manufacturer). The use of this column solved the tailing problem and under our experimental conditions phenazines separated from each other according to their polarity properties.

Supernatants of bacterial cultures contain a lot of polar substances. Most of them could be separated from phenazines by extensive elution with solvent A (15 min) and 10% of solvent B (10 min). We tried to reduce these times by modifying solvent composition, but all of the assayed changes resulted in a poorer separation of pyocyanin (not shown).

We analyzed phenazines in the supernatants (aged for two weeks at room temperature) from cultures of *P. aeruginosa* grown in both KA and MM media at 30°C for 20 h. Typical chromatograms of supernatant samples are shown in Figs. 3 and 4. The peaks corresponding to the pigments could be separated from peaks corresponding to other compounds contained in the sample. Moreover, the retention times of the pigments were practically the same as those found for standard phenazines run under identical conditions (Fig. 1).

These results indicate an optimum method per-

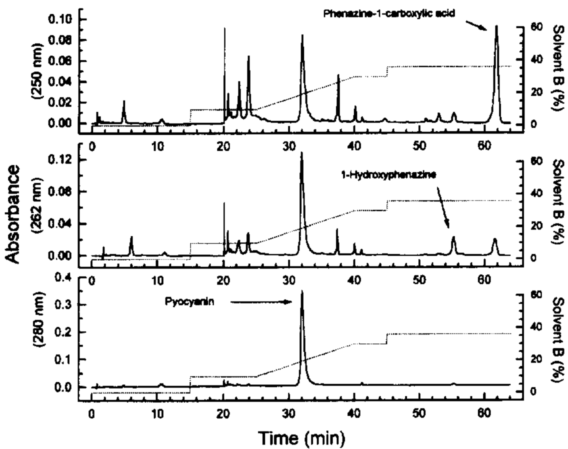

Fig. 4. HPLC analysis of supernatant of *P. aeruginosa* cultured in a simple medium (MM). Wavelengths employed for monitoring are indicated in parentheses at ordinate axes. Samples of 50 μl were injected.

formance, since the mentioned phenazines could be separated not only from non-phenazine compounds, but also from other phenazines such as phenazine and phenazine methosulfate (data not shown).

3.4. Limit of detection and quantification

We have calculated limits of detection and limits of quantification (QL) taking into account the total error affecting the measure, as previously described [26,27]. Limits of quantification of standards were 2.1 ng for phenazine-1-carboxylic acid, 7.5 ng for 1-hydroxyphenazine and 5 ng for pyocyanin, when wavelengths at the maximum absorption peak of the spectra were used. Similar results were obtained when samples of cultures were analyzed. Since chromatograms of samples from LB and KA culture media showed an unknown peak very close to the pyocyanin peak (without complete separation), it was necessary to monitor at 390 nm in those cases to eliminate interferences. This caused a decrease of sensitivity, but it is not important since the QL value (13 ng) is much lower than the typical concentration of pyocyanin found in biological samples.

3.5. Calibration curves, recovery and precision

Regression analysis of examples of calibration curves for standards and for complex samples spiked with known quantities of standards are shown in Table 2.

Correlation coefficients were higher than 0.999 in all cases and areas measured by triplicate samples at each point differed by an average of less than 1% indicating a good precision for this method.

Under our experimental conditions (no controlled temperature) retention times were reproducible within 0.5 min.

Recoveries were determined as described in Section 2. Although the values for standard added at 1 $\mu g\ ml^{-1}$ was less than those for standard added at 12.5 $\mu g\ ml^{-1}$, the results indicate that good recoveries were reached. In fact, recoveries for the three compounds were about 90% and 100% for standard added at 1 $\mu g\ ml^{-1}$ and 12.5 $\mu g\ ml^{-1}$, respectively. Furthermore, standard deviations were in general less than 1% of the calculated concentrations suggesting again that the method presents a highly acceptable precision.

The present method offers a rapid and reliable analysis of phenazine pigments of *P. aeruginosa* either in complex or non-complex samples. The advantage of our HPLC procedure over previously published techniques is that it presents high resolution of several phenazines including pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxylic acid, phenazine and phenazine methosulfate (the latter two not shown); moreover, it avoids the comigration observed with the previously described method when complex samples were studied. This assay should facilitate the determination of the role of these substances in pathogenesis and in the investigation on metabolic aspects in the production of *Pseudomonas* phenazines.

Table 2
Phenazine calibration curves

	Phenazine-1-carboxylic acid		1-Hydroxyphenazine		Pyocyanin	
	Standards	Sample spiked with standards	Standards	Sample spiked with standards	Standards	Sample spiked with standards
Interception term (mAU)	4.26 (3.19)	61.74 (2.57)	−1.21 (3.92)	45.27 (2.17)	0.56 (1.63)	62.46 (0.47)
Slope (mAU μg^{-1})	266.8 (2.5)	261.1 (2.2)	283.4 (3.0)	278.1 (1.8)	67.2 (1.2)	67.3 (0.4)
Regression coefficient	0.99987	0.99986	0.99983	0.99991	0.99947	0.99993
Standard deviation	4.64	4.64	5.91	3.92	2.46	0.85
Number of points	5	6	5	6	5	6

The HPLC column was loaded with amounts of standards ranging from 0.1 to 2.5 μg. The same amounts of standards were used to spike sample.

Linear regression analysis was obtained from the average of three values of areas at each point for standards and for samples of supernatant of *P. aeruginosa* LB cultures spiked with standards. Values in parentheses indicates the standard deviation of the corresponding parameter.

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