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Quantitative analysis of a bacteria-derived antibiotic in nematode-infected insects using HPLC–UV and TLC–UV methods

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Abstract

3,5-Dihydroxy-4-isopropylstilbene (ST), an antibiotic produced by the bacterial symbiont *Photorhabdus luminescens* of the nematodes of the genus *Heterorhabditis* was determined quantitatively in nematode bacterium-infected insects using HPLC or TLC for separation and UV for quantification. Comparable and reproducible results were obtained with both HPLC–UV and TLC–UV methods. Several factors, including solvents for extraction of the antibiotic from the infected insects, eluents for TLC development and programs for HPLC operation, were investigated. Of the four solvents used, namely acetone, methanol, ethyl acetate and diethyl ether, acetone had the highest extraction efficacy, and the ST recovery rate was about 95%. ST can be easily separated from all other bacterial metabolites on a TLC plate using a mixture of chloroform–methanol (98.5:1.5) or by HPLC using acetonitrile and water as the mobile phase.

Keywords: 3,5-Dihydroxy-4-isopropylstilbene

1. Introduction

Nematodes of the genus *Heterorhabditis* carry a luminous, bacterial symbiont, *Photorhabdus luminescens*, in their gut [1]. These parasitic nematodes penetrate insects and expel their bacterial symbionts into the insect hemocoel [2,3]. Once released, the bacteria multiply rapidly, the insect dies within 24–48 h, and the nematodes feed on the multiplying bacteria. The insect cadaver becomes deep red but does not putrefy, apparently because of antibiotic(s) produced by the bacteria [4–9]. Two antibiotics, namely, 3,5-dihydroxy-4-isopropylstilbene (ST) (Fig. 1) and 3,5-dihydroxy-4-ethylstilbene, have been identified following their isolation from a culture broth of *P. luminescens* [7], and ST has been reported as present in the culture broths of all of the several different strains of *P. luminescens* studied [7–9]. Further, ST is active not only against bacteria [7], but also against several fungal [9] and nematode

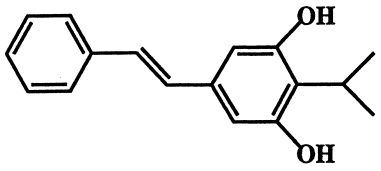

Fig. 1. Structure of 3,5-dihydroxy-4-isopropylstilbene.

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species [10]. Consequently, ST probably plays an important role in the biological relationship between these bacteria, nematodes and insects. In order to fully understand the role of this antibiotic in this biological interaction, and its potential biological control application in agriculture, it was necessary to determine the amount of ST quantitatively in the parasitized insect cadaver. This paper reports on two reliable and readily available methods based on high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) for separation, and subsequent ultraviolet (UV) detection for the quantification of ST.

2. Experimental

2.1. Materials

Entomopathogenic nematodes, *Heterorhabditis megidis* strain 90, were originally collected from soil in British Columbia, Canada. They were maintained in this laboratory in last-instar larvae of the Great Wax Moth, *Galleria mellonella*, that were reared [12] and supplied by the insectary of the Department of Biological Sciences, Simon Fraser University [11]. Infective juveniles (IJs) were collected on a water trap within 4 days of their initial emergence from the insect cadaver [11]. IJs that passed through two layers of wet-strength tissue into distilled water were collected, washed and surface sterilized with 0.2% thimerosal (Sigma) [11], and the suspension was adjusted to 6250 nematodes/ml of phosphate-buffered saline (PBS) [12] prior to injection into the insect larvae. For each experiment, larvae of similar weight were injected with the nematodes (25 IJs/larva) in 4 μl of PBS and kept at 25°C in the dark.

The standard chemical, ST, was prepared [9] and further purified by HPLC. Its structure was confirmed by spectroscopic analysis. All other chemicals used in the study were either HPLC or analytical grade.

2.2. Spectral analysis

Low resolution mass spectra were obtained on a Hewlett-Packard 5985B GC–MS system operating at 70 eV using a direct probe. The UV spectra of ST and UV absorbance of each test sample were obtained on a Milton 3000 UV spectrometer.

2.3. HPLC analysis

HPLC analysis was performed using a Waters 626 liquid chromatograph. The mobile phase was acetonitrile and water, which was filtered and sparged prior to use. The mobile phase was delivered at 1.2 ml/min to a 250×4.6 mm Nucleosil 5 C_{18} column (Phenomenex, Rancho Palos Verdes, CA, USA) with the following program: 15% MeCN in water for 1 min, followed by a linear gradient to 62% MeCN in water in 20 min, and isocratic (62% MeCN in water) for 5 min. The eluate was passed through a Waters 484 tunable absorbance detector set at 315 nm. The results of the analysis were recorded with a Waters 746 data module.

2.4. TLC analysis

A 250 ml beaker containing 20 ml of the developing solvent was sealed with aluminium foil for TLC development. Kieselgel 60 F254 TLC aluminium sheets (Merck, Darmstadt, Germany) were used for all the TLC analyses and cut into small plates (9× 2.5 cm). The sample was applied as a band 1 cm from the bottom of the plate and the solvent was allowed to migrate to 1 cm from the top. A mixture of methanol–chloroform (1.5:98.5) was selected as the developing solvent. Disposable 10 μl micropipettes (Drummond Scientific, Broomall, PA, USA) were used to quantitatively transfer samples to the TLC plates. The samples on the TLC were visualized with a UV lamp (254 nm). The ST band was completely separated from the other bands, cut off selectively, and the piece then immersed immediately in 1 ml of methanol in a 1.5 ml centrifuge tube. The resulting solution was put aside for 1 h in the dark, stirred for 3 min on a deluxe mixer and centrifuged for 3 min (13 000 *g*) to give a clear supernatant, which was then transferred to a 1.5 ml disposable UV grade cuvette with two optical windows (VWR Scientific of Canada, Edmonton, AB, Canada), and used directly for UV absorbance measurement by a Milton 3000 UV spectrometer. In some cases, the above supernatant was diluted with

methanol first before measuring for UV absorbance measurement.

2.5. Standard curves

A 4 mg amount of ST was dissolved in 2 ml of methanol to generate the stock solution with a concentration of 2000 μg/ml. The stock solution was then serially diluted by half with methanol to give a series of solutions with concentrations of ST ranging from 1000 μg/ml to 0.122 μg/ml. 20 μl of each standard solution were injected for HPLC analysis, and 0.8 ml was used each time for UV absorbance measurement.

The standard curve of ST for HPLC analysis was established. The following equation, $C=0.150+4.494\times A$, was obtained for those standard solutions with concentrations of ST from 1.95 μg/ml to 62.5 μg/ml; where A=the area recorded (recorded units/100 000, with a wavelength of 315 nm set for the detector), and C=the corresponding concentration of the antibiotic (μg/ml) in the sample injected into the HPLC system with a fixed volume of 20 μl. With a total of six points, the coefficient of regression was 0.9999, standard error 0.304, standard deviations of the slope and intercept were 0.026 and 0.172, respectively.

The standard curve of ST for TLC analysis was established. The equation, $C=-0.021+8.878\times A$, was obtained for those standard solutions with concentrations of ST from 0.122 μg/ml to 15.625 μg/ml; where A=the absorbance (wavelength: 315 nm), and C=the corresponding concentration of the antibiotic (μg/ml) in the test sample. With a total of eight points, the coefficient of regression was 1.0000, standard error 0.021, and the standard deviations of the slope and intercept were 0.013 and 0.009, respectively.

2.6. Effects of solvents on the recovery efficacy of ST extraction

Three replicates, each of five cadavers of *G. mellonella*, were selected randomly from those infected with IJs 3 days after injection and used for each solvent extraction. Four solvents, namely acetone, ethyl acetate, diethyl ether and methanol, were tested following the same extraction procedure. Each replicate group of five larvae was immersed in a mortar containing 3 ml of the specific solvent and homogenized. The resulting liquid extract was transferred into a 25 ml flask. The residues were re-extracted with 1 ml of the same solvent four times and centrifuged at 13 000 *g* when necessary. All the extracts were combined in the same 25 ml flask and dried under vacuum below 30°C. The dried material was dissolved with 1 ml of methanol, transferred to a centrifuge tube and centrifuged (13 000 *g*). The supernatant was poured off, diluted 100-times with methanol, and 20 μl of the diluted solution was subjected to HPLC analysis in order to determine the recovery efficacy of the test solvents on ST extraction.

2.7. Recovery efficacy of ST extraction using acetone

Two standard ST solutions, with concentrations of 25 μg/μl and 2.5 μg/μl, were prepared by dissolving ST in dimethylsulfoxide (DMSO). Each of five healthy larvae of *G. mellonella* was injected with 5 μl of a standard ST solution and immediately immersed in a mortar containing 3 ml of acetone. All the five larvae (all of which had been injected in less than 1 min) were homogenized together. The same extraction procedure was followed as described in Section 2.6. The supernatant of the extract was first diluted with methanol 50-times (for the larvae injected with standard solution with a concentration of 25 μg/μl) or 10-times (for the larvae injected with standard solution with a concentration of 2.5 μg/μl) to fit the linear range of the standard curve established for HPLC analysis, then analyzed to determine the recovery efficacy. 20 μl of each diluted sample was injected each time. The study was repeated three times.

2.8. Comparison of the methods developed

Cadavers of *G. mellonella* were selected randomly from among those larvae that 2 days or 5 days earlier had been infected by IJs. Acetone was used as the solvent for extraction, and the same procedure as described above was used to prepare the corresponding supernatants. Ten μl of each supernatant were delivered to the TLC plate for analysis, fol-

lowed by the TLC procedure described in Section 2.4. Before HPLC analysis, the supernatants from the larvae infected for 2 days were diluted 50-times with methanol and the supernatants from the larvae infected for 5 days were diluted 100-times with methanol. Twenty μl of each of the resulting solutions were analyzed by HPLC.

3. Results and discussion

3.1. HPLC analysis

Initially, when the UV detector was set at 254 nm, many peaks were detected (Fig. 2), and so their separation was optimized. Once the gradient was chosen for HPLC analysis, following several trials with different gradients, the detector was reset at 315 nm, the wavelength with the maximum absorbance for ST (Fig. 3). This change increased the sensitivity of the UV detection. The peak for ST was identified by comparing it with that of a standard sample, and further verifying it by UV and MS spectral data [7–9].

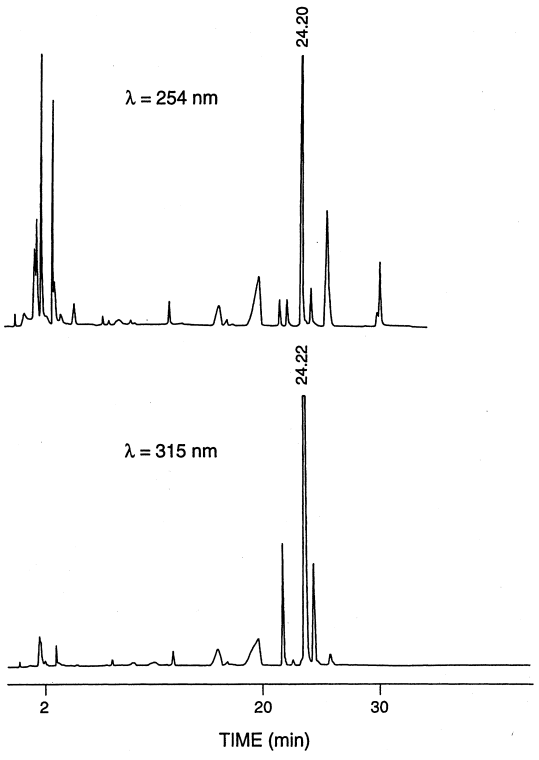

Fig. 2. Comparison of HPLC chromatograms of a typical test sample extracted from *Galleria mellonella* larvae infected with *Heterorhabditis megidis* strain 90, as detected at two different wavelengths (254 nm and 315 nm).

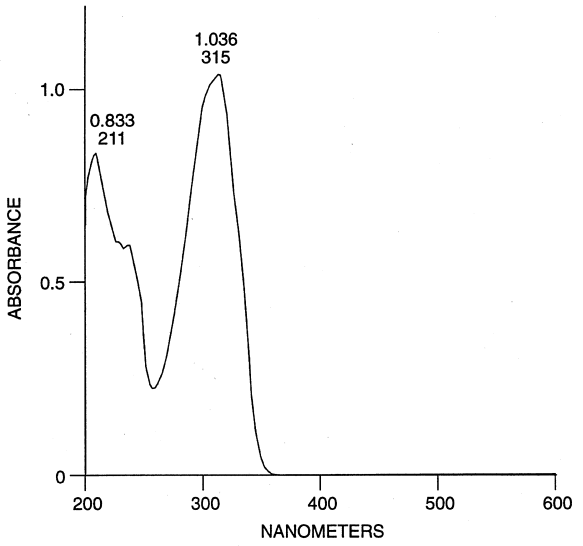

Fig. 3. UV spectrum of 3,5-dihydroxy-4-isopropylstilbene.

3.2. TLC analysis

The nature of the developing solvent was extremely important in order to obtain complete separation of ST from the other substances. Initially, hexane and ether were used [9], but the band of ST was mixed with another band. However, it was observed that with a mixture of methanol and chloroform the separation efficacy was very sensitive to the ratio of methanol and chloroform. After many trials, a ratio of 1.5:98.5 for the methanol–chloroform mixture was selected for all analyses (Fig. 4). This ratio gave complete separation of ST from all other metabolites in all samples tested. The R_F for the ST antibiotic was 0.59, while those for the closest bands were 0.74 and 0.45. After the TLC plate was developed with the selected developing solvent, the amount of ST in the TLC plate should be detectable with a UV scanner, but such a scanner was not available. Consequently, an alternative quantifying method with a common UV spectrometer was developed. The ST band was cut off selectively, and immersed

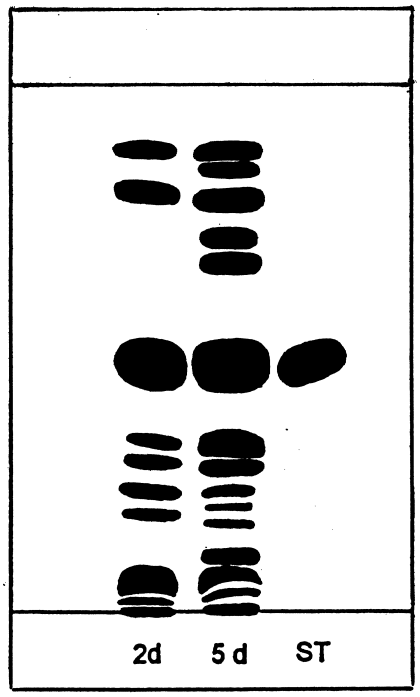

Fig. 4. TLC chromatogram of 3,5-dihydroxy-4-isopropylstilbene (ST) and two test samples extracted from *Galleria mellonella* larvae infected with *Heterorhabditis megidis* strain 90, 2 and 5 days (2d and 5d) after infection. Note the differences in the associated bands due to the changing of biological process in insect–nematode–bacterium complex over time. TLC plate (9× 5.3 cm) was developed under the same conditions as stated in Section 2.4 and visualized under UV lamp (254 nm).

in 1 ml of methanol in a 1.5 ml centrifuge tube. Extraction of ST from the TLC piece was rapid and efficient, and the amount of ST extracted from the TLC piece after its immersion in methanol for 30 min was determined to be the same as that obtained after 60 min with stirring. Conservatively, all the samples in the experiments were put aside in methanol for 1 h, stirred for 3 min on a deluxe mixer and then centrifuged for 3 min to give clear supernatants for quantification. In order to fit the linear part of the standard curve, the supernatants were transferred, undiluted or diluted with methanol, to 1.5 ml disposable, UV grade cuvettes with two optical windows for UV absorbance measurement. The control solution used for UV measurements was either methanol or a methanol extract from a blank TLC piece obtained by a method similar to those for the test samples, and there was no difference observed for the two control solutions.

3.3. Standard curves

The standard curves for both HPLC and TLC analysis were repeated on two different days, and the results were the same. Consequently, the two methods are validated with excellent repeatability.

3.4. Recovery efficacy of ST extraction

Based on the area recorded for the HPLC analysis of each replicate, the concentration of the injected solution can be calculated from the standard HPLC curve. Since the injected solution was prepared by diluting the original sample with methanol 100-times, the total amount of ST extracted from each replicate was obtained by multiplying the concentration and volume of the original sample prepared (1 ml). During the experiment, five larvae of *G. mellonella* were used in order to allow for the variation among samples, although one cadaver could be expected to have sufficient amount of ST antibiotic to be analysed. The results are summarized in Table 1. Acetone extracted more ST than the other

Table 1
Extraction of 3,5-dihydroxy-4-isopropylstilbene (ST), using different solvents, from *Galleria mellonella* infected with *Heterorhabditis megidis* strain 90[a]

Solvent	Wet weight (g) of five larvae	Area of HPLC analysis	Amount (μg) of ST extracted	Amount (μg) of per gram of larvae
Ethyl acetate	0.659±0.019	3.82±0.27	1732±123.30	2632±243.55
Diethyl ether	0.713±0.034	1.85±0.20	848±87.23	1187±92.05
Methanol	0.681±0.011	2.94±0.40	1337±180.85	1956±289.16
Acetone	0.710±0.024	4.84±0.33	2192±149.50	3085±111.97

[a]Data is expressed as the mean±S.D. (n=3).

Table 2
Recovery of 3,5-dihydroxy-4-isopropylstilbene (ST) extracted with acetone from healthy *Galleria mellonella* larvae injected with known amounts of the substance[a]

Total ST (μg) injected into five insect larvae	Area recorded by the HPLC method (dilution)	ST (μg) present in five insect larvae as determined by the HPLC method	Percentage of ST recovered
0[b]	0	0	
62.5	1.31±0.03 (10)	60.43±1.54	96.7±2.47
625	2.61±0.11 (50)	593.67±25.48	95.0±4.09

[a]Data is expressed as the mean±S.D. (n=3).
[b]DMSO control.

solvents tested and, therefore, was selected as the solvent for extraction in subsequent experiments.

Once acetone was selected to be the solvent for extraction, the recovery efficacy of acetone for extracting ST from the insect larvae was studied. The amount of ST injected into each of the five, healthy *G. mellonella* larvae was fixed at either (25 μg/μl× 5 μl=) 125 μg or (2.5 μg/μl×5 ml=) 12.5 μg, resulting in a total amount of ST in the five larvae of 625 μg or 62.5 μg. The amount of ST injected into the insects was then extracted and determined using the HPLC method described above, and the results are summarized in Table 2. The recovery rates were about 95%, thus fully proving the reliability of this extraction process. When the amount of ST injected into five larvae was increased to more than 1000 μg, a similar, high recovery rate (95%) was obtained.

3.5. Comparison of the methods developed

Both the TLC and HPLC methods described above were applied to the analysis of ST in *G. mellonella* larvae that had been infected by the nematodes 2 days and 5 days previously. The results are summarized in Table 3. In each gram of both 2 day and 5 day nematode infected larvae, the mean amount of ST present as determined by the HPLC method was 1504 μg and 4067 μg, respectively, and as determined by the TLC method was 1434 μg and 3942 μg, respectively. The data obtained with both TLC and HPLC methods were comparable (within 5%), and, therefore, proved the reliability and applicability of both methods. By having two satisfactory methods available, experimenters are able to select the most appropriate one based on the availability and convenience of suitable, analytical equipment.

4. Conclusions

Based on the above results, two effective methods of quantifying ST in insect larvae have been developed that are based on the ready availability of HPLC, TLC and UV analytical equipment. The two

Table 3
Level of 3,5-dihydroxy-4-isopropylstilbene (ST) present in *Galleria mellonella* larvae infected with *Heterorhabditis megidis* strain 90, as determined by HPLC and TLC methods[a]

Content	Days after infection	HPLC	TLC
Wet weight (g) of five larvae at sampling	2	1.036±0.013	1.036±0.013
	5	0.987±0.006	0.987±0.006
Area or absorbance (dilution)	2	6.91±0.71 (50)	0.85±0.03 (2)
	5	8.89±0.32 (100)	0.44±0.01 (10)
ST (μg) determined in five larvae	2	1559±159.49	1502.33±51.42
	5	4011±144.22	3889±109.19
ST per gram of larvae (μg/g)	2	1503.67±137.16	1434.33±66.34
	5	4065.67±161.74	3942.00±123.22

[a]Data is expressed as the mean±S.D. (n=3).

methods are reliable, data repeatable, and the equipment is usually available to those in the fields. Consequently, the methods are useful tools to those needing to determine the presence of this substance in living systems, such as in the bacterium–nematode–insect complex. A similar approach could be developed for other bioactive compounds obtained from the symbiotic bacteria of entomopathogenic nematodes. The methods could be useful also in the development of ST and its derivatives as agrochemicals.

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References

[1] G.O. Poinar Jr., G. Thomas, M. Haygood, K.H. Nealson, Soil Biol. Biochem. 12 (1980) 5.
[2] G.O. Poinar, G.M. Thomas, Parasitology 56 (1966) 385.
[3] G.O. Poinar Jr., Nematologica 21 (1975) 463.
[4] R.J. Akhurst, J. Gen. Microbiol. 128 (1982) 3061.
[5] P.A.D. Grimont, A.G. Steigerwalt, N. Boemare, F.W. Hickman-Brenner, C. Deval, F. Grimont, D.J. Brenner, Int. J. Syst. Bacteriol. 34 (1984) 378.
[6] O.N. Morris, Can. Entomol. 117 (1985) 401.
[7] V.J. Paul, S. Frautsch, W. Fenical, K.H. Nealson, J. Chem. Ecol. 7 (1981) 589.
[8] W.H. Richardson, T.M. Schmidt, K.H. Nealson, Appl. Environ. Microbiol. 54 (1988) 1602.
[9] J. Li, G. Chen, H. Wu, J.M. Webster, Appl. Environ. Microbiol. 61 (1995) 4329.
[10] K. Hu, J. Li, J.M. Webster, Can. J. Plant Pathol. 18 (1996) 104.
[11] G. Chen, G.B. Dunphy, J.M. Webster, Biol. Control 4 (1994) 157.
[12] P.W. Maxwell, G. Chen, J.M. Webster, G.B. Dunphy, Appl. Environ. Microbiol. 60 (1994) 715.