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Quantitative determination of latrunculins A and B in the Red Sea sponge *Negombata magnifica* by high performance liquid chromatography

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Abstract

An accurate, reproducible and sensitive method for the quantitative determination of latrunculins A and B in the organic extract of the Red Sea sponge *Negombata magnifica* was developed and validated. Latrunculin A and B concentrations were determined by RP-C18-HPLC and a mobile phase consisting of acetonitrile and water (60:40, v/v). The flow rate utilized was 1 mL min^{-1} and the detector was set at 235 nm. The HPLC analysis of several *N. magnifica* samples collected from different locations in the Red Sea revealed that Ras Mohamed had the highest concentrations of latrunculin A, while Safaga had the highest levels of latrunculin B. Also, a comparison between latrunculin concentrations in the summer and winter revealed that the yield of latrunculins were generally higher in the winter.

Keywords: *Negombata magnifica*; Red Sea; Latrunculin A; Latrunculin B; Season; Location; High performance liquid chromatography

1. Introduction

The macrolides, latrunculin A and B, were first isolated from the Red Sea sponge *Negombata magnifica* (formerly *Latrunculia magnifica*) [1]. Among the characteristic features of these natural molecules is the presence of a macrocyclic lactone ring structure of 14 or 16 carbon atoms as well as a 2-thiazolidinone moiety (Fig. 1) [2,3]. Latrunculins A and B were later isolated from taxonomically unrelated sponges [4–7]. In addition to the pronounced ichthyotoxic properties of latrunculins, they were found to be cytotoxic and also possess antiviral activity. In vitro experiments revealed that the latrunculins disrupt actin filaments [8,9]. The latrunculins have been shown to alter cell shape [10]; disrupt actin microfilament organization [8,11]; inhibit the microfilament-mediated processes of meiosis [12], fertilization and early development [13]; force development in muscles [14]; and even affect protein kinase C signaling [15]. These results have raised interest in the potential use of latrunculins as growth inhibitors of some tumor cell lines, and therefore, the possibility for them to serve as prototypes in the discovery and development of novel antitumor agents. The interest in latrunculin as a viable lead for the treatment of cancer is further extenuated by the compounds structural homology with the epothilones which are currently in clinical development as controls for a number of different cancer types.

This work represents the first report on the simultaneous quantitative determination of latrunculin A and B in the organic extracts of *N. magnifica* from different locations in the Red Sea in different seasons. The HPLC method was developed to provide a specific procedure for the rapid and facile analysis of an extract containing latrunculins A and B. The method was also used for quantitative analysis of latrunculins in order to determine the time (season) and optimal location for collection in order to produce the maximum amounts of latrunculins A and B without interference from other compounds in the extract. The proposed HPLC method succeeded to separate latrunculin A from B, although with thin layer chromatography they have almost identical rates of flow (R_f) [16].

The HPLC method described herein is rapid and accurate and utilizes instrumentation that is readily available in most laboratories. We propose that this method will help in identifying the best time of the year as well as the optimal location for har-

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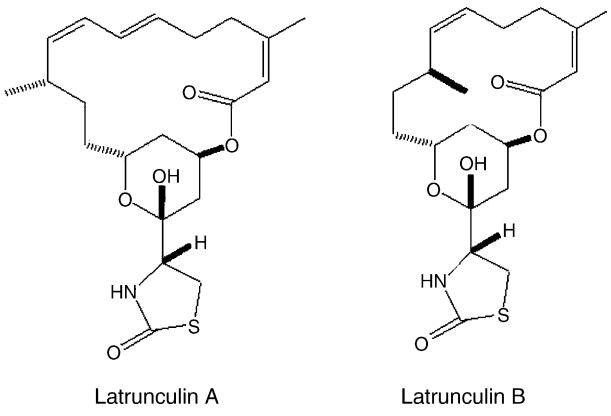

Fig. 1. Structure of latrunculins A and B.

vesting sponges producing latrunculins A and B. It may also be used to monitor aquaculture experimental conditions for large-scale production and isolation of these medicinally valuable compounds.

2. Experimental

2.1. Instruments

The LC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 μl loop and a SPD-10AVP UV–vis detector. Separation and quantitation were made on a 250 mm × 4.6 mm (i.d.) 5 μm ODS column (Luna, Phenomenex, USA). The detector was set at λ 235 nm. Data acquisition was performed on class-VP software.

A double-beam Shimadzu (Kyoto, Japan) UV–vis spectrophotometer, model UV-1601 PC equipped with 1 cm quartz cells and connected to an IBM compatible computer. The bundled software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth was 2 nm and the wavelength scanning speed was 2800 nm min^{-1}.

2.2. Materials and reagents

2.2.1. Animal material

Samples of the sponge *N. magnifica* were collected by hand using SCUBA from different locations in the Red Sea. Three colonies (replicates) were collected from each location: Sharm El-Sheikh (SH), Ras Mohamed (RM), Nabq (NA), Hurghada (HU), Safaga (SA), Quser (QU) and Tiran straits (TS). These samples were collected in the Summer (S) June 2004 and Winter (W) January 2005. The samples were frozen immediately after collection on site and kept frozen until analyzed.

2.2.2. Latrunculins A and B

Latrunculin A was isolated by the fractionation of a portion of the organic extract of the sponge *N. magnifica* collected from Sharm El-Sheikh on a silica gel column using 15% ethyl acetate in hexane as an eluent. The fraction containing latrunculins was further purified by sephadex LH-20 using methanol:chloroform (1:1). The identity of the isolated latrunculin A was confirmed by comparing its NMR spectra with published data [2].

Latrunculin B was isolated by the fractionation of a portion of the organic extract of the sponge *N. magnifica* collected from the Hurghada area on a silica gel column using 15% ethyl acetate in hexane as an eluent. The fraction containing latrunculins was further purified by sephadex LH-20 using methanol:chloroform (1:1). The identity of the isolated latrunculin B was confirmed by comparing its NMR spectra with published data [17].

2.2.3. Chromatographic materials and solvents

Pre-coated silica gel G-25 UV_{254} plates for thin layer chromatography (20 cm × 20 cm) (E. Merck, Germany), Silica gel 60/230–400 μm mesh (E. Merck, Germany) and Sephadex LH-20 (Sigma–Aldrich, USA) for column chromatography were used.

The solvents used in this work were chromatographic grade acetonitrile, methanol, *n*-hexane, ethyl acetate (Fisher, UK), dichloromethane (BDH, AnalaR, UK) and distilled water.

2.3. Chromatographic conditions

The HPLC separation and quantitation were made on a 250 mm × 4.6 mm (i.d.) 5 μm ODS column (Luna, Phenomenex, USA). The mobile phase was prepared by mixing acetonitrile and water in a ratio of 60:40 (v/v). The mobile phase was filtered using 0.45-μm membrane filter (Millipore, Milford, MA) and degassed by vacuum prior to use. The flow rate was 1 mL min^{-1}. The samples were also filtered using 0.45 μm disposable filters. The injection volume was 20 μL. All determinations were performed at ambient temperature. The detector was set at the wavelength of maximum absorption (235 nm). This was determined by dissolving 0.5 mg latrunculin B in 50 mL mobile phase. The UV absorption spectra of standard solutions of latrunculin B were recorded in 1 cm quartz cell over the range 200–350. Data acquisition was performed on class-VP software.

2.4. Standard solutions and calibration

Stock solution of latrunculin A was prepared by dissolving 5 mg in 5 mL mobile phase (stock working solution). Also, stock solution of latrunculin B was prepared by dissolving 5 mg in 5 mL mobile phase (stock working solution).

The standard solutions were prepared by dilution of stock working solution with the mobile phase to reach the concentration range of 20–100 μg mL^{-1} for latrunculins A and B. Triplicate 20-μL injections were made for each concentration and chromatographed under the conditions described above (Figs. 2 and 3). The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph.

2.5. Sample preparation

The frozen samples were freeze dried and then extracted with methanol, dichloromethane mixture (1:1) for 5 days. The solvent

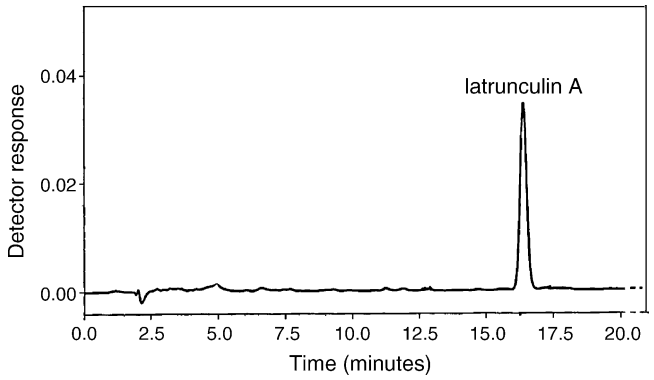

Fig. 2. A typical chromatogram of 20-μL injection of latrunculin A (10 mg% at 235 nm).

mixture was replaced everyday to ensure extraction efficiency. The extract was dried under vacuum. Complete extraction was confirmed by thin layer chromatography and high performance liquid chromatography.

2.5 mg of the extract were dissolved in 10 mL of the mobile phase (60% acetonitrile, 40% water) in a volumetric flask producing a final concentration of 25 mg%. The contents of the flasks were shaken vigorously for 10 min, sonicated for 15 min then filtered through 0.45 μm disposable filters and 20 μL injection of each sample was assayed.

2.6. Statistical analysis

Data were presented as mean ± S.E. (Table 2). The data were computed using SPSS program and analyzed by one-way analysis of variance (ANOVA) followed by PostHoc test (Bonfreroni) for multiple comparisons of latrunculin concentrations. Student's *t*-test was used for statistical comparison between latrunculin concentrations in the summer and winter. Significant results were determined as $p < 0.05$.

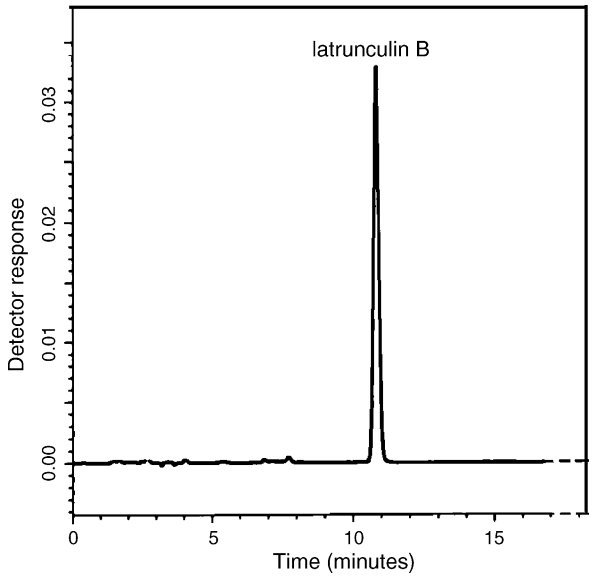

Fig. 3. Atypical chromatogram of 20-μL injection of latrunculin B (10 mg% at 235 nm).

3. Results and discussion

3.1. HPLC method

A single, isocratic, selective reverse-phase liquid chromatographic method has been developed for the simultaneous determination of latrunculins A and B. In this method, a satisfactory separation was obtained with a mobile phase consisting of acetonitrile and water in a ratio of 60:40 (v/v). Quantitation was achieved with UV detection at 235 nm based on peak area. The average retention time ± standard deviation (S.D.) was found to be 16.3 ± 0.3 min for latrunculin A and 10.8 ± 0.13 min for latrunculin B. The selectivity of the RP-HPLC is illustrated in Fig. 4 where there is good separation of latrunculins A and B form the rest of the components of the extract in the different locations.

3.2. Validation

3.2.1. Linearity

The linearity of the proposed method was evaluated by analyzing a series of different concentrations of latrunculin A and B. In this study, five concentrations were chosen, ranging from 20 to100 μg mL^{-1} for each of latrunculin A and latrunculin B using the proposed HPLC method. Each concentration was repeated three times. The assay was performed according to experimental conditions previously established. The linearity of the calibration graphs and adherence of the system to Beer's law were validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p < 0.05$) different from zero (Table 1).

3.2.2. Range

The calibration range was established through consideration of the practical range necessary according to latrunculin A and B concentrations present in sponge extracts to give accurate, precise and linear results. The calibration range is given in Table 1.

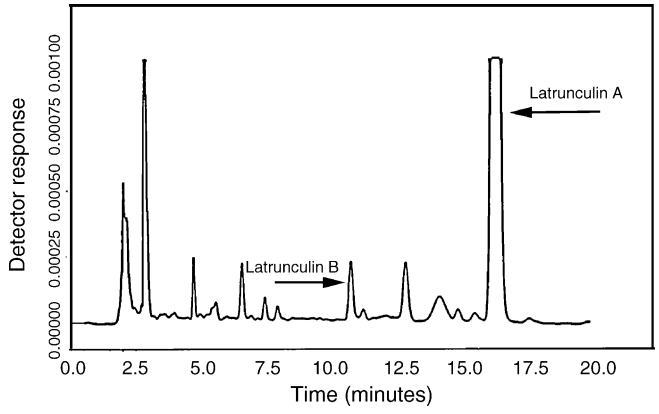

Fig. 4. Chromatogram showing the chromatographic separation of latrunculins A and B from other organic compounds of *N. magnifica* from Ras Mohamed at 235 nm.

Table 1
Assay parameters and regression characteristic of latrunculins A and B determined by HPLC

Parameters	HPLC	
	Latrunculin A	Latrunculin B
Calibration range (μg/mL)	20–100	20–100
Regression equation (Y)[a]		
Slope (b)	264611	111504
Intercept (a)	92411	−40190.2
Standard deviation of the slope	13356.9	7848.6
Standard deviation of the intercept	90393.8	50256
Confidence limit of the slope[b]	245601.4–283621.4	100333.5–122674.4
Confidence limit of the intercept	−36240.5–221062.6	−111716.2–31335.83
Correlation coefficient	0.99923	0.99985
Standard error of the estimation	36236.3	21292.88

[a] $Y = a + bC$, where C is the concentration of drug in $\mu g\,mL^{-1}$ and Y is the peak area in the HPLC method.
[b] 95% confidence limit.

Table 2
Concentration of latrunculins A and B in the in the dry weight calculated from the regression equation

Location	Season	Latrunculin A in dry weight (%)	Latrunculin B in dry weight (%)
Ras Mohamed (RM)	Summer (S)	3.17 ± 0.0028	0.024 ± 0.0005
Sharm El-Sheikh (SH)	Summer (S)	2.20 ± 0.0078	0.112 ± 0.0017
Sharm El-Sheikh (SH)	Winter (w)	2.15 ± 0.002	0.22 ± 0.0072
Nabq (NA)	Summer (S)	0.0032 ± 0.00005	1.78 ± 0.0044
Nabq (NA)	Winter (w)	0.0225 ± 0.00008	1.98 ± 0.0076
Hurghada (HU)	Summer (S)	0.0057 ± 0.00015	1.51 ± 0.0057
Hurghada (HU)	Winter w)	0.0027 ± 0.00011	2.08 ± 0.0076
Safaga (SA)	Summer (S)	0.0025 ± 0.000014	1.90 ± 0.011
Safaga (SA)	Winter (w)	0.020 ± 0.00057	2.16 ± 0.0044
Quseir (QU)	Summer (S)	0.004 ± 0.00015	1.82 ± 0.0044
Quseir (QU)	Winter (w)	0.0052 ± 0.00011	2.14 ± 0.0057
Tiran straits (TS)	Summer (S)	3.10 ± 0.03	0.42 ± 0.0028

3.2.3. Precision

For evaluation of precision and repeatability, three concentrations of the authentic latrunculin A and B were assayed over several days. There was no significant difference in the mean results obtained from one day to another.

3.2.4. Selectivity

The HPLC method was selective for latrunculin A and B. It was able to detect latrunculin A and B in the complex natural extract with minimal interference from other compounds in the extract.

Peak purity was confirmed by collection of eluted peaks of latrunculin A and B followed by comparing nuclear magnetic resonance spectra of the eluted peaks with that of pure standards isolated from the sponge.

3.2.5. Accuracy

The study was performed by the addition of known amounts of the authentic latrunculin A and B to a sample with a known minimal amount of latrunculin A and B. The mixture was assayed and results were obtained and compared to the expected values. The results revealed an increase in the peak area in response to the added latrunculin A and B.

3.2.6. Stability

The *N. magnifica* extract and the authentic latrunculins A and B dissolved in the mobile phase (60% acetonitrile and 40% water) were stable at room temperature for up to 1 weak. No chromatographic changes were observed.

3.2.7. Statistical analysis

Ras Mohamed was shown to be the location with the highest concentration of latrunculin A. The difference between Ras Mohamed and all other locations was statistically significant ($p < 0.05$). Safaga was found to be the location with the highest concentration of latrunculin B. The difference between Safaga and all other locations except Quser was statistically significant ($p < 0.05$). The winter was generally a better season for the isolation of latrunculins. The difference between the winter and the summer was found to be significant in all locations.

4. Conclusion

To the authors knowledge, this is the first report on an HPLC method for the quantitative and qualitative analysis of latrunculins. It provides a simple, accurate and reproducible quantitative analysis for separation and determination of latrunculin

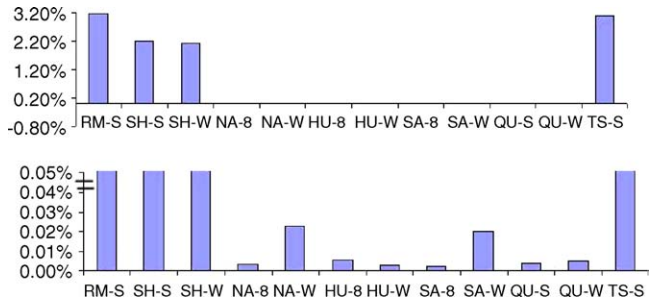

Fig. 5. The concentration of latrunculin A in dry weight of *N. magnifica* from different locations and seasons.

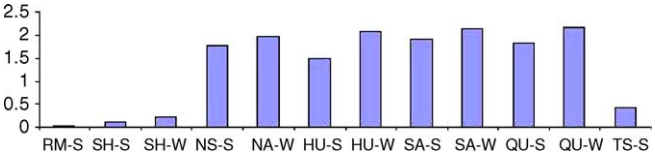

Fig. 6. The concentration of latrunculin B in dry wight of *N. magnifica* from different locations and seasons.

A and B concentrations in natural extracts without interference from other compounds in the extract.

It has been reported elsewhere that sponges from the Gulf of Aqaba produce latrunculin B alone, while those from the western side of the Sinai Peninsula near Sharm El-Sheikh and in the Gulf of Suez contain only latrunculin A. In the Tiran straits, however, the sponges were reported to possess either latrunculin A or latrunculin B (but not both within the same individual) [16].

The present work using the proposed analytical method demonstrates that latrunculin A and B are present in all samples form all locations. While one location may contain one of the latrunculins as a major component, a small amount of the other latrunculin can still be detected using the HPLC method described in this paper. Therefore, samples from the locations that were sampled in this study produce both latrunculin A and latrunculin B. Locations with high yields of latrunculin A were found to produce minor amounts of latrunculin B and vice versa (Fig. 4 and Table 2).

Of the locations sampled in this study, our HPLC method found Ras Mohamed to be the location with the highest concentration of latrunculin A. Safaga was shown to be the location for sponges with the highest concentration of latrunculin B (Figs. 5 and 6). A possible explanation may come from the fact that latrunculin A is more potent than latrunculin B [18]. Therefore, the locations in which there is more stress may produce the substance that is more toxic. Ras Mohamed, being rich in its biodiversity, the competition level is high. There is also heavy human impact due to boating and diving activities. The reef in the location of collection is also very much exposed. On the other hand, the Safaga area of collection has much less human impact, biodiversity and competition and the reef is much more sheltered. Accordingly, there is much more stress in Ras Mohamed than there is in Safaga. This may at least partially explain the reason for the difference in concentrations of the individual latrunculins in the different locations.

Also, our findings show that the winter is generally a better season for production of latrunculins. This may be explained by the fact that *N. magnifica* undergoes reproduction in the summer [16]. Further studies are required to determine the optimal environmental conditions for the production of latrunculins A and B.

Since latrunculins represent interesting prototype compounds with significant anticancer activity, it is expected that semisynthetic derivatization may lead to a new anticancer agent for the pharmaceutical industry. In this case, the supply of latrunculins will be an important question to answer. The HPLC method described in this paper will serve as a useful monitoring tool in studying the effects of genetics as well as environmental conditions on the production of latrunculins. This should prove valuable as large-scale production of these important compounds through aquaculture experiments may be necessary to meet the needs of the pharmaceutical industry.

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