Development and Validation of a New HPLC Method for the Determination of Biphenyl and Dibenzofuran Phytoalexins in Rosaceae

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Abstract

A simple, precise, rapid and accurate isocratic reversed-phase high-performance liquid chromatographic method was developed and validated for the simultaneous determination of biphenyl (aucuparin and noraucuparin) and dibenzofuran (eriobofuran) phytoalexin from elicitor treated cell culture of Sorbus aucuparia (mountain ash). These phytoalexins play crucial role in combating scab disease in many commercially important rosaceous plants, such as apple, pear and mountain ash. The isocratic separation was performed in a Luna C18 reversed-phase column (250 × 4.6 mm, 5 μm particle size) using a mobile phase of 1 mM trifluoroacetic acid (TFA) in water with methanol [40:60 (v/v)]. Quantization of phytoalexin was carried out on Shimadzu-HPLC system using a Photo Diode Array (PDA) detector at 254 nm by comparing the peak area. Peak purity and identity were confirmed by UV spectroscopy and ESI–MS-MS in the negative ion mode. The different analytical performance parameters such as linearity, accuracy, precision, limit of detection and limit of quantification were determined according to the International Conference on Harmonization guidelines. Linearity was observed in the concentration range of 3–400 µg/mL with excellent correlation coefficient ($R^2 ≥ 0.995$). This newly developed method is rapid, easy, cost-effective and can be used for monitoring scab-resistance potential of rosaceous plants.

Introduction

Malinae subtribe of rosaceae is well known for bearing a number of economically important fruit plants such as apple (Malus domestica), pear (Pyrus species) and quince (Cydonia oblonga) (1). Malinae fruits are prized for their unique textures and flavors as well as their health promoting properties. In 2013, the global production of apple was accounted 80 million tones, out of which 2.6% were contributed by the Indian (FAOSTAT 2015). However, dramatic losses in fruits and trees are caused by two apple diseases; apple scab caused by the fungus Venturia inaequalis and the fire blight, caused by the bacterium Erwinia amylovora (2). Apple cultivars are mostly selected by vegetative propagation of single trees, leading toward genetic uniformity in commercial apple orchards, which seriously affect their resistance toward pathogen and diseases (3). Upon pathogen attack, the Malinae forms two specialized class of phytoalexins, biphenyls and dibenzofurans (4). The ability to produce these two classes of inducible defence compounds is confined to the members of Malinae only (1). These phytoalexins are produced upon pathogen infection or after elicitor challenge. Till date, 10 different types of biphenyls and 17 different types of dibenzofurans have been reported from various genera of Malinae (5). Previously, it was assumed that biphenyls and dibenzofurans do not occur simultaneously (6). Nonetheless, recently it has been demonstrated that elicitor-treated cell cultures of Sorbus aucuparia and scab-resistant apple cultivars accumulate both biphenyls and dibenzofurans simultaneously (1, 7), which suggested a biosynthetic relationship between these two classes of compounds. Interestingly,
scab-susceptible apple cultivars failed to produce these phytoalexins (8). These phytoalexins from Malinae have gained immense attention due to their antibacterial and antifungal properties, and their presence has been shown to be closely related to scab-disease tolerance of apples. These antimicrobial phytoalexins inhibit the growth of pathogens during plant–pathogen interactions. In the Malinae, the most abundant biphenyl phytoalexins are aucuparin and noraucuparin, whereas eriobofuran represent most important dibenzofuran phytoalexin (5). The content of these inducible phytoalexins varied widely in different apple cultivars depending on factors such as genotype, climate and pathogen infection. These phytoalexins in apples and pears serve as marker compounds for scab and fire-blight tolerance. In order to obtain scab or fire blight-resistant apples and pears for commercial plantation, it is essential to rapidly determine the level of these phytoalexins in different cultivars of apples and pears. Till date, analysis and characterization of biphenyl and dibenzofuran phytoalexins from Malinae has relied mainly on the gas chromatography (GC) based method (1). However, the use of GC presents a number of difficulties including the instability of the derivatized phytoalexin and the self-destructive analytical process, hence not being suitable for preparative work. This study describes a rapid HPLC-based method coupled with diode array detection (DAD) and mass-spectrometry for the simultaneous detection and identification of biphenyl and dibenzofuran phytoalexins from cell cultures of S. aucuparia. Identification of each phytoalexin was accomplished by comparison of retention time (RT) and UV spectral properties with known standards. The chemical identity of aucuparin, noraucuparin and eriobofuran was further confirmed by ESI–MS–MS analyses. The method can easily be extended to the routine analysis of apple phytoalexins to determine their scab-tolerance. The main advantage of this method is the rapid separation and quantification of noraucuparin, aucuparin and eriobofuran, which are among the most important phytoalexins of Malinae. Furthermore, the monitoring of the level of these phytoalexins is essential for the assessment of pathogen defense potential of Malinae plants.

Experimental

Plant material

Cell suspension cultures of S. aucuparia (mountain ash; Malinae) served as an in vitro system for this study. The cells were grown and treated with Venturia inaequalis preparation to induce phytoalexin formation as described previously (2, 9).

Reagents and chemicals

Analytical grade chemicals were used in sample preparation. HPLC grade solvents were used for HPLC analyses. Aucuparin, noraucuparin and eriobofuran were synthesized as described previously (2). Trifluoroacetic acid was obtained from Loba chemicals (Mumbai, India). Methanol, acetonitrile and ethyl acetate were obtained from SDFCL (Mumbai, India).

HPLC conditions

HPLC analyses were performed on a Shimadzu-HPLC system (Shimadzu Corporation, Kyoto, Japan), equipped with a CBM-20A controller, an LC-20AP pump and a SPD-M20A Photo Diode Array (PDA) detector. The sample was injected through a Rheodyne™ 7725i injection valve with a 20-µL sample loop. Chromatographic separation was achieved on a Luna C18 reversed-phase column 250 × 4.6 mm, 5 µm particle size (Phenomenex, Torrance, CA, USA) coupled with a Phenomenex Security Guard™ C18 guard column 4 × 3 mm (Torrance, CA, USA).

The mobile phase was degassed as well as vacuum filtered through 0.45 µm nylon membranes. The mobile phase consisted of an isocratic solvent mixture comprising 1 mM trifluoroacetic acid (TFA) in water–methanol [40:60; (v/v)] with a flow rate of 0.5 mL/min. The detection wavelength was set at 254 nm. Peaks were identified by comparing their RT and UV spectra with those of authentic standards. Data were acquired and processed with LC-Solution software (Shimadzu Corporation, Kyoto, Japan) on Windows 7™ platform. Peaks from the plant samples corresponding to aucuparin, noraucuparin and eriobofuran were eluted and subjected to electron spray ionization mass spectrometry (ESI–MS–MS) under negative ion mode for the confirmation of their chemical identity.

ESI–MS conditions

ESI–MS analyses were carried out on a QTrap 3200 system (Applied Biosystems/MDS Sciex). HPLC elutes were directly introduced to an ESI source (Turbo V; Applied Biosystems/MDS Sciex) using a Hamilton syringe pump and a flow rate of 5 µL/min. The mass spectrometer was operated in the negative mode with a source voltage and declustering potential of −4.5 kV and −20 V, respectively. Nitrogen gas was used for nebulization, with the curtain gas, gas 1 and gas 2 settings at 10, 14 and 0, respectively. Putative product molecular ion peaks [M–H]− were further analyzed by MS–MS experiments in the enhanced product ion mode of the instrument using nitrogen gas for collision-induced dissociation at the high-level setting. The collision energy was set at −21 V. Compounds were identified by comparison with their reference standards. Data were acquired and processed with Analyst software (version 1.4.2; Applied Biosystems/MDS Sciex).

Preparation of standards and plant sample

Standard stock solutions of 1 mg/mL of each of aucuparin, noraucuparin and eriobofuran were prepared in aqueous methanol (50:50, v/v). Working standard solutions in the range of 1.5–400 µg/mL were prepared by dilution of standard stock solution with aqueous methanol (50:50, v/v). All stock solutions were stored at −20°C. For the sample preparation, 5-day old S. aucuparia cell culture was treated with Venturia elicitore preparation (50 mg/L) to induce phytoalexin formation. Forty-eight hours after the treatment, cells were collected by filtration and 2 g fresh cell mass was extracted in 4 mL of methanol. The extract was recovered by centrifugation at 10,000 rpm for 20 min. The supernatant was collected, filtered through 0.45 µm filters (Millipore, Bangalore, India) and directly used as the sample for HPLC analyses.

Calibration procedure

Nine different concentrations were used to prepare the standard calibration plot for noraucuparin, aucuparin and eriobofuran. Noraucuparin and aucuparin were used in the concentration range of 1.5–400 µg/mL, whereas eriobofuran was used in the range of 3–400 µg/mL. The working concentration of reference compounds were achieved by diluting the stock solutions with aqueous methanol (50:50, v/v). Each calibration solution was injected into HPLC in triplicate. The calibration curve was prepared by plotting the peak area against the concentration of the compound. The calibration curves, characterized by slope, intercept and correlation coefficient, were used to determine the concentration of the respective analyte in the sample, limit of detection (LOD), limit of quantification (LOQ) and analytical sensitivity (AS).

Recovery, precision, accuracy and specificity

The relative recoveries were determined by the method of standard addition. Homogenized elicited cell culture of S. aucuparia was spiked
with three known concentrations of each of the three standards (50, 100 and 200 µg/mL) before following the described extraction procedure. Three replicate analyses were run for all the three standards at defined concentrations, and the peak area ratios of three extracted sample solutions at each concentration level were compared with the standard solutions to get the percent recovery. These samples were analyzed the next day to obtain inter-day precision. The precision was expressed in terms of relative standard deviation (RSD %). The accuracy was expressed as the percentage of the analyte concentration measured in each sample relative to the known amount of the analyte spiked to the sample.

AS, LOD and LOQ
Analytical sensitivity was calculated according to ALAMIN program as describe before (10). Analytical sensitivity was expressed as the ratio of $S/n$, where $S$ is the residual standard deviation and $n$ is the slope of the calibration curve. The limit of detection (LODapprox) and the limit of quantification (LOQapprox) are determined by the following equations:

$$\text{LOD}_{\text{approx}} = 3 \times (\text{AS}) \times \left( \frac{n-2}{n-1} \right)^{1/2},$$

$$\text{LOQ}_{\text{approx}} = 10 \times (\text{AS}) \times \left( \frac{n-2}{n-1} \right)^{1/2},$$

where $n$ is the number of total measurement for each calibration set and AS is the analytical sensitivity.

Results
Optimization of chromatographic conditions
Various isocratic combination of acetonitrile–water or methanol–water were tried to achieve optimum chromatographic conditions for noracuparin, aucuparin and eriobofuran. Optimum separation with good peak shapes was observed in the mobile phase comprising of 1 mM TFA in water and methanol in the ratio of 40:60 (v/v). The optimized flow rate was found to be 0.5 mL/min. Under these optimized conditions, the chromatograms of standard noracuparin, aucuparin and eriobofuran were obtained as shown in Figure 1. A good separation was achieved within 40 min with the above-described chromatographic conditions. The RTs for noracuparin, aucuparin and eriobofuran were 20.0, 34.1 and 39.9 min, respectively.

Method validation
The HPLC method was validated for linearity (see calibration), LOD, LOQ, accuracy, precision and peak purity. The method was validated according to International Conference on Harmonization guidelines (11).

Linearity
Excellent linearity was observed between the peak area and concentration in the range of 3–400 µg/mL for noracuparin and aucuparin and of 6–400 µg/mL for eriobofuran. The coefficient of regression of the calibration curve obtained for all the three analytes were more than 0.995, thereby confirming the linearity of the developed method. The parameters of the linear regression equation for each analyte are presented in Table I.

AS, LOD and LOQ
The results for the AS, LOD and LOQ are listed in Table II. The LOD was found to be 0.76 µg/mL for noracuparin, 0.40 µg/mL for aucuparin and 3.77 µg/mL for eriobofuran. The corresponding limits of quantification were found to be 2.5, 1.3 and 12.6 µg/mL, respectively. These results clearly indicate that the analytical method has good sensitivity.

Precision and accuracy
The precision and accuracy data for noracuparin, aucuparin and eriobofuran are presented in Table III. The method was found to be
Table I. Regression Parameters of the Calibration Curve \((n = 3)\)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear range (µg/mL)</th>
<th>(y = bx + a^a)</th>
<th>Correlation coefficient ((R^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noraucuparin</td>
<td>1.5–400</td>
<td>(y = 716.8x + 387.1)</td>
<td>0.999</td>
</tr>
<tr>
<td>Aucuparin</td>
<td>1.5–400</td>
<td>(y = 620.7x + 459.3)</td>
<td>0.999</td>
</tr>
<tr>
<td>Eriobofuran</td>
<td>6–400</td>
<td>(y = 61.7x + 187.5)</td>
<td>0.996</td>
</tr>
</tbody>
</table>

\(^a y = \text{peak area}; x = \text{concentration.}\)

Table II. Performance Characteristics

<table>
<thead>
<tr>
<th>Compound</th>
<th>AS (µg/mL)</th>
<th>LOD(_{\text{approx}}) (µg/mL)</th>
<th>LOQ(_{\text{approx}}) (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noraucuparin</td>
<td>0.35</td>
<td>0.76</td>
<td>2.50</td>
</tr>
<tr>
<td>Aucuparin</td>
<td>0.18</td>
<td>0.40</td>
<td>1.30</td>
</tr>
<tr>
<td>Eriobofuran</td>
<td>1.77</td>
<td>3.77</td>
<td>12.6</td>
</tr>
</tbody>
</table>

AS, analytical sensitivity; LOD, limit of detection; LOQ, limit of quantification.

Table III. Recovery, Precision (Intra- and Inter-Day) and Accuracy (%) Data for the Simultaneous Determination of Noraucuparin, Aucuparin and Eriobofuran

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked analyte (µg/mL)</th>
<th>Recovery (µg/mL)</th>
<th>Intra-day precision (% RSD)</th>
<th>Accuracy (%)</th>
<th>Inter-day precision (% RSD)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noraucuparin</td>
<td>50</td>
<td>50.50 ± 0.96</td>
<td>1.90</td>
<td>101.01</td>
<td>52.17 ± 0.34</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>104.03 ± 0.13</td>
<td>0.12</td>
<td>104.03</td>
<td>103.16 ± 0.31</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>203.95 ± 1.58</td>
<td>0.77</td>
<td>101.98</td>
<td>207.58 ± 3.51</td>
<td>1.70</td>
</tr>
<tr>
<td>Aucuparin</td>
<td>50</td>
<td>52.04 ± 0.42</td>
<td>0.81</td>
<td>104.08</td>
<td>52.33 ± 0.89</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>102.84 ± 0.23</td>
<td>0.22</td>
<td>102.85</td>
<td>103.86 ± 2.63</td>
<td>2.53</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>202.62 ± 0.77</td>
<td>0.38</td>
<td>101.31</td>
<td>204.99 ± 0.39</td>
<td>0.19</td>
</tr>
<tr>
<td>Eriobofuran</td>
<td>50</td>
<td>47.92 ± 0.72</td>
<td>1.50</td>
<td>95.85</td>
<td>48.62 ± 0.25</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>102.33 ± 3.90</td>
<td>3.82</td>
<td>102.33</td>
<td>103.00 ± 3.72</td>
<td>3.61</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>203.34 ± 3.65</td>
<td>1.79</td>
<td>101.67</td>
<td>206.03 ± 1.24</td>
<td>0.60</td>
</tr>
</tbody>
</table>

\(\% \text{RSD}, \text{relative standard deviation.}\)

Figure 2. HPLC chromatogram of the elicited cell culture of \(S. \text{aucuparia}\). HPLC conditions are the same as described in ‘Experimental’ section. Detection wavelength 254 nm.
Discussion

Disease resistance mechanism of Malinae plants such as apples, pears, mountain ash toward scab disease is poorly understood. Upon fungal infection, scab-resistant cultivars of apples and cell cultures of mountain ash are known to produce two special classes of phytoalexins, biphenyl and dibenzofuran (2, 5, 8). Pathogen defense potential of Malinae plants are determined by their ability to produce these two classes of marker phytoalexins. Till date only GC–MS based method is available for the simultaneous determination of these two classes of phytoalexins. However, such methods are not suitable for preparative work due to self-destruction of metabolites during GC-based analyses (12). In this study, a validated HPLC method was established for rapid quantification of aucuparin, noraucuparin and eriobofuran phytoalexins from cell cultures of mountain ash with a simple and accurate method. The RT for noraucuparin, aucuparin and eriobofuran was observed to be 20, 34.1 and 39.9 min, respectively. Peak identities were further determined by ESI–MS analyses in negative mode. The proposed method demonstrated high specificity at 254 nm for the detection of phytoalexins and high reliability in the quantification of noraucuparin, aucuparin and eriobofuran. Moreover, the method has the following advantages: rapid extraction, isocratic run condition, no need for equilibration between two successive runs and easy sample preparation. Taking into account the results obtained in this study, the proposed method can be conveniently used for the analysis of similar class of scab-induced marker phytoalexins from economically important rosaceous plants such as apples and pears.

Conclusion

HPLC analyses coupled with a PDA detector and mass spectrometry proved to be an efficient method for the simultaneous detection and quantification of biphenyl and dibenzofuran phytoalexins from rosaceous plants. The evaluated validation characteristics showed that the technique is very simple, sensitive, reproducible and accurate. The detection limits for the measured compounds are excellent. The sample extraction and preparation process prior to analysis is very simple and fast, which could be applied to nearly any type of plant materials containing biphenyl and dibenzofuran phytoalexins. Furthermore, the separation was performed under isocratic conditions; thereby, no re-equilibration is required in between two successive HPLC runs.

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