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Rapid separation of antimicrobial metabolites by microchip electrophoresis with UV linear imaging detection

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Abstract

This research examines microchip electrophoresis with linear imaging UV detection for the analysis of antimicrobial metabolites, monoacetylphloroglucinol (MAPG) and 2,4-diacetylphloroglucinol (2,4-DAPG) from *Pseudomonas fluorescens* F113. Initial results show the separation of MAPG, 2,4-DAPG and resorcinol in less than 20 s. This was achieved using a quartz microchip with a separation channel length of 25 mm. In order to quantitate the amount of MAPG and 2,4-DAPG in a microbial cultured supernatant sample, on-chip sample introduction in a methanol/buffer matrix was investigated. Sample introduction/injection parameters were optimized to improve sensitivity and thus decrease the limit of detection (LOD). The amount of antimicrobial metabolites present was quantitated with a separation time of 15 s. A previously developed capillary electrophoretic method was compared to the microchip method in relation to speed, efficiency, precision, linear range and limit of detection. This investigation shows the fastest separation so far of these antimicrobial metabolites with high efficiency.

Keywords: Monoacetylphloroglucinol (MAPG); 2,4-Diacetylphloroglucinol (2,4-DAPG); Antimicrobial metabolites; Microchip electrophoresis (MCE); *Pseudomonas fluorescens* F113

1. Introduction

The human genome project has left a legacy of interest in gene expression. This has resulted in intense research focus in areas such as genomics, proteomics and metabolomics. The metabolome includes hundreds of low-molecular-weight compounds that play vital roles in relation to the complex biochemical and metabolic processes occurring within cells and biological fluids. Metabolites are chemically diverse compounds that are released in a wide concentration range [1]. The term secondary metabolite [2] was first mentioned in the early 1960s to indicate microbial metabolites found as products of differentiation in restricted taxonomic groups and not necessary for metabolism.

Microchip electrophoresis (MCE) [3] is an emerging new technology that epitomises the current trend towards miniaturization of chemical systems. The main advantages include the capability of assaying complex multicomponent matrices in record time periods of a few seconds. Microchips have the potential to challenge more traditional columns and capillaries due to speed, size, versatility, portability, reduced solvent and sample consumption and lower cost. The ability of a microchip platform to deliver high-speed separations and thus high sample throughput has allowed this format to deliver some of the fastest separations reported to date [4]. Constant demands within the pharmaceutical, clinical and biotechnology sectors for rapid, cheaper, highly efficient separations have catalyzed the need for these miniaturized separation techniques and devices.

Phloroglucinols have many important roles to play in both medicine and agriculture. Release of antimicrobial agents by microorganisms, in particular phloroglucinols, is recognized as a significant biocontrol mechanism for inhibiting plant disease and death. The polyketide metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) is made by many strains of fluorescent *Pseudomonas* spp. with a major function being biocontrol of soil-borne fungal plant pathogens [5]. While monoacetylphloroglucinol (MAPG) possesses antibacterial properties, 2,4-DAPG displays stronger potency [6].

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2,4-DAPG plays a major role in the biological control of numerous plant-destroying diseases. This phenolic compound has broad-spectrum antibacterial and antifungal properties [7]. As with many natural products, phloroglucinol (benzene-1,3,5-triol) is used in medicine as a muscle relaxant, as it shows no anticholinergic potency and appears to have lower toxicity than other antispasmodic agents [8]. To date, chromatographic methods have played an important role in the analysis of phloroglucinol compounds. Lartigue-Mattei et al. employed a method for determining phloroglucinol in human plasma by gas chromatography–mass spectrometry [9]. Similar studies by Kim et al. resulted in a sensitive and selective liquid chromatography–mass spectrometric (LC–MS) method for determining phloroglucinol in plasma samples [8]. Bonsall et al. have reported antibiotic isolation from both soil and broth cultures with further quantitation by reversed-phase high-performance liquid chromatography [10]. Shanahan et al. had previously published a reversed-phase liquid chromatographic analysis of 2,4-DAPG in culture and soil samples [11]. Later, Shanahan et al. employed a gradient LC assay for determining MAPG and 2,4-DAPG in growth culture media [12]. More recently, Picard and Bosco studied 2,4-DAPG production from different strains of *Pseudomonas* using [13], a method previously described by Keel et al. [14]. Kamei and Isnansetyo published findings of a novel antibacterial mode of action of 2,4-DAPG against methicillin-resistant *Staphylococcus aureus* (MRSA) [15]. Recently, Siddiqui and Shaukat revealed the importance of the bacterial metabolite 2,4-DAPG in the suppression of root-knot disease in tomatoes [16].

This work investigates the rapid separation of resorcinol along with metabolites MAPG and 2,4-DAPG on a quartz microchip with UV linear imaging detection. UV detection eliminates the need for sample derivatization and is more convenient than the commonly used laser induced fluorescence detection systems [17]. Typical separation parameters varied during optimization, included voltage, buffer concentration and pH, and sample introduction and separation times. A sub-study of different methods of on-chip sample introduction was also undertaken. A previously developed CE method [18] is compared with the microchip method in relation to speed, efficiency, precision, and limit of detection (LOD). A microbial cultured supernatant sample from *Pseudomonas fluorescens* F113 was for the first time analyzed by MCE, and its metabolite content determined in 15 s.

2. Experimental

2.1. Instrumentation

All electrophoretic separations were performed using a Shimadzu microchip electrophoresis (MCE) 2010 system (Shimadzu GmbH, Duisburg, Germany). The detection system was a UV linear imaging system (190–370 nm) under normal polarity settings (towards the cathode). The operating system was Windows 2000 and the software was MCE-2010. The system utilised a 0–1800 V high-voltage supply. The Shimadzu quartz microchip employed for all separations have dimensions of 30 μm width × 30 μm depth with a total separation/analytical channel length of 25 mm. There were four platinum electrodes on the chip to apply voltages between the sample introduction and separation reservoirs, the chip were encased in a polypropylene frame. The quartz microchip was rinsed between separations using an inbuilt automatic buffer wash. If blockage was suspected, the microchip was rinsed with 0.1 M NaOH using a hand-held plastic syringe for 15 min. At the end of each day, an inbuilt automatic water-flushing programme was switched on so that the microchip would not become dry. At all times, the microchip was stored in the MCE instrument. On applying voltages between the microchip reservoirs, SI and SO refer to sample inlet and outlet positions, while BI and BO refer to the buffer inlet and outlet.

2.2. Reagents

Sodium tetraborate (borax), phloroglucinol (anhydrous) and boron trifluoride diethyl etherate complex (for the synthesis of 2,4-DAPG) were purchased from Sigma–Aldrich (UK). HPLC grade methanol was purchased from Labscan Ltd. (Dublin, Ireland). HC1 and NaOH were purchased from E. Merck (Darmstadt, Germany). Tetrahydrofuran (THF) was purchased from Labscan Ltd. (Dublin, Ireland). Resorcinol (99%) was purchased from Sigma–Aldrich (Dublin, Ireland). 2,4-DAPG was synthesized in our laboratories according to the Dean and Robertson procedure [19]. MAPG (98%) was purchased from Sigma–Aldrich (Dublin, Ireland) under the trade name 2,4,6-trihydroxyacetophenone monohydrate. Dual filter membranes (0.45 μm) were purchased from Millipore Ltd. (Cork, Ireland). Disposable plastic syringes (2 mL) were purchased from Lennox Laboratory Supplies (Dublin, Ireland). A plastic 1 mL syringe unit with an inbuilt filter for manually rinsing the microchip was supplied with the microchip courtesy of Shimadzu (Duisburg, Germany).

2.3. MAPG and 2,4-DAPG standard preparation

Standard solutions in the range of 0.5–400 mg/L MAPG and 2,4-DAPG were prepared in 50% sodium tetraborate (25 mM, pH 9.3) and 50% methanol for calibration and method development studies. These standard solutions had a final concentration of 12.5 mM sodium tetraborate. In addition, a stock solution of MAPG, 2,4-DAPG and resorcinol (500 mg/L) was prepared in 100% sodium tetraborate (50 mM, pH 9.3), for analysis by pinched sample introduction. All solutions, buffers and samples were filtered through dual filter membranes (0.45 μm), and stored in a fridge.

2.4. Bacterial strains and growth conditions

The bacterial stains examined were *P. fluorescens* F113, originally isolated from sugar-beet rhizosphere [20] and *P. fluorescens* F113 phloroglucinol-negative derivative G22 [21]. Both stains were routinely grown in sucrose asparagine (SA) medium supplemented with 100 μM $FeCl_3$ at 28 °C [22]. Cultures were inoculated at an $OD_{600\,mm}$ 0.01 and growth monitored every 4 h. Bacterial supernatant samples were taken 12 h post-inoculation for analysis by MCE.

2.5. Solid-phase extraction

Solid-phase extraction (SPE) of bacterial supernates was carried out using a Sep-pak C_{18} cartridge for the purpose of sample cleanup as described previously [20]. Briefly, at defined time-points throughout bacterial growth, 1.5 mL of supernate was sampled and bacterial cells removed by centrifugation at 3000 rpm. Supernatant samples were syringe forced through a Sep-pak cartridge as just described. The cartridge was then washed with 20 mL of deionised water of Milli-Q grade with a resistivity of 18.2 MΩ cm, and the phloroglucinol derivatives eluted with 2 mL HPLC grade methanol. Subsequently, these methanol extracts were diluted with an equal volume of sodium tetraborate (25 mM, pH 9.3), as described in Section 2.3. The final concentration of sodium tetraborate was 12.5 mM.

2.6. Electrolyte preparation

A 25 mM stock sodium tetraborate running buffer solution (pH 9.3) was prepared by dissolving 4.7675 g in 500 mL of Milli-Q grade water (resistivity of 18.2 MΩ cm). For pH variation of the sodium tetraborate buffer, a 100 mM borate stock solution was diluted with Milli-Q grade water and acid or base to a final concentration of 25 mM. The pH was adjusted using 1 M NaOH or 1 M HC1 in the pH range 8.0–9.5. For variation of run buffer concentration (10–20 mM), a 100 mM borate stock solution was diluted with Milli-Q grade water and the pH was checked. All running buffers were sonicated (ULTRAsonik NEY) for 10 min to remove dissolved air, and filtered using dual filter membranes (0.45 μm).

3. Results and discussion

3.1. Initial studies on the separation of resorcinol, MAPG and 2,4-DAPG

Initial results involving the separation of resorcinol, MAPG and 2,4-DAPG using pinched sample introduction are shown in Fig. 1. The order of elution (resorcinol, 2,4-DAPG and MAPG) is determined by the degree of anionisation and molecular weight with MAPG migrating the slowest, and thus eluting last. For pinched sample introduction, the separation time was 17 s, whereas for gated sample introduction the optimum separation time was 15 s. Separation using the pinched method displayed theoretical plate values of 1579 (MAPG), 2157 (2,4-DAPG) and 3639 (resorcinol), calculated per total separation channel length (25 mm), according to Eq. (1):

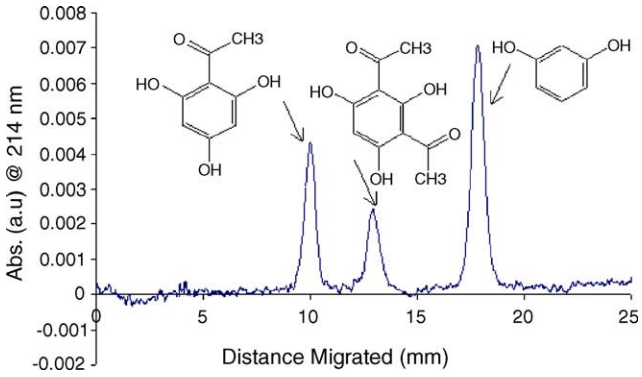

Fig. 1. Microchip electrophoretic separation of MAPG, 2,4-DAPG and resorcinol. Sample introduction conditions: sample inlet (SI) 1 kV, sample outlet (SO) 0 kV, buffer inlet (BI) 0.7 kV, buffer outlet (BO) 0.9 kV for 35 s. Separation settings: SI 0.5 kV, SO 0.5 kV, BI 0.7 kV, BO 0 kV for 17 s. Detection at 214 nm, running electrolyte: sodium tetraborate (25 mM, pH 9.3); MAPG, 2,4-DAPG and resorcinol were dissolved in 100% sodium tetraborate (50 mM, pH 9.3). Total analysis time 52 s (sample introduction and separation time).

$$N = \left(\frac{d_m}{SS}\right)^2 = 2\pi\left(\frac{d_m h}{A}\right)^2 \quad (1)$$

where SS is the variance of band in distance (mm), d_m the distance migrated from cross-channel to band center (mm), h the peak height (uabs), and A the peak area (a.u. × 60) (a.u. = absorption units).

3.2. Effect of variation of run buffer concentration on resolution and migration time

At 10 and 15 mM borate running buffer, the analytes remained unresolved due to the high velocity of the EOF. Moreover, due to the limited separation channel length, the EOF at 20 and 25 mM allowed the ions more time to resolve in a timescale of seconds due to the lower velocity. The optimum separation conditions were found to be 25 mM sodium tetraborate running electrolyte at pH 9.3.

3.3. Effect of variation of pH of run buffer on resolution

The pH of the sodium tetraborate electrolyte was varied in the pH range 8.0–9.3. The velocity of the EOF was lowest at 8.0 resulting in co-elution of MAPG and 2,4-DAPG. At pH 8.5, MAPG and 2,4-DAPG were just partially resolved. As the buffer pH was increased to 9.0 and upwards, two separate bands were observed, MAPG becomes increasingly anionic and this lead to baseline resolution. A working pH of 9.3 was chosen, as no pH adjustment was necessary. Resolution was best at pH 9.5, a value of 3.0 was noted.

3.4. Effect of variation of voltage for sample introduction and separation

For pinched sample introduction, the sample inlet voltage was varied from 1.0 to 1.4 kV. At higher inlet voltages (1.3 and 1.4 kV), the analytes were unresolved possibly due to overloading as a result of the high concentration. The optimum settings for pinched sample introduction were 1.0 kV at the sample inlet with the outlet grounded while simultaneously applying 0.7 kV to the buffer inlet and 0.9 kV to the buffer outlet for 35 s. The gated approach is discussed in Section 3.5.

The separation voltage was also varied (0.7–1.7 kV). For quantitative analysis, the optimum separation voltage was 1.7 kV. As diffusion is a limiting factor for efficient separations, high electric field strength will limit diffusion time and thus give highest efficiency. On increasing separation voltage, the number of theoretical plates increased in a linear fashion according to the equation [23]:

$$N = \frac{\mu_{ep} E L}{2 D_m} \quad (2)$$

where N is the number of theoretical plates, μ_{ep} the solute mobility, E the electric field strength, L the total capillary length, and D_m the solvent diffusion coefficient.

3.5. Optimization of gated sample introduction for real sample analysis

As microbial cultured supernatant samples were subjected to SPE with subsequent elution in methanol, analyte introduction in a methanol matrix was investigated. Standard MAPG and 2,4-DAPG solutions were prepared in a 50:50% MeOH–borate buffer mix (12.5 mM). Depending on the stage of the bacterial growth cycle, metabolites are released in varied high and low concentrations. In order to decrease the limit of detection, the sample introduction parameters were further varied. In order to introduce a large analyte plug, gated sample introduction was investigated. The optimum voltage settings were sample inlet (SI) at 1 kV while grounding the sample waste (SO), and simultaneously holding the buffer inlet (BI) at 0.34 kV and the buffer outlet (BO) at 0.62 kV (Fig. 2). Under these conditions, a high separation voltage was chosen (1.7 kV) at the buffer inlet (SI), while the buffer outlet (BO) was grounded, with the sample inlet (SI) and outlet held at 0.50 kV for 15 s separation time. Using these conditions, the LOD was approximately 4 mg/L for MAPG, and 5 mg/L for 2,4-DAPG by inspection of the signal being three times that of the noise, on increasing the sample introduction time to 20 s. However, no difficulty in detection sensitivity was encountered, as this method can easily accommodate a preconcentration step during SPE if required.

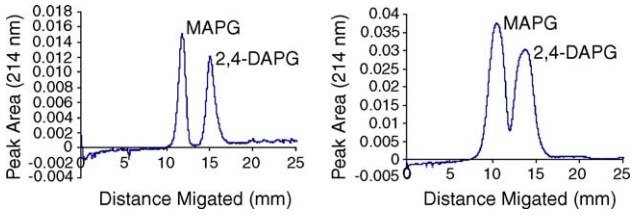

Fig. 2. Chip electropherogram showing the effect of gated sample introduction at two different voltage settings: (a) 1 kV; (b) 1.3 kV. Total analysis time 30 s. All other conditions as in Table 1.

As these conditions would have led to a small amount of sample entering the buffer inlet (BI), a linearity and precision study was carried out to see how well the sample volume could be controlled. The sample introduction time was kept short (15 s) for standard solutions of MAPG and 2,4-DAPG ranging from 10 to 100 mg/L. Ten replicate runs of each standard were used for statistical analysis. The standards were made up as described in Section 2.3 for method development studies. The correlation coefficients obtained for both compounds were greater than 0.98, with R.S.D. values for migration times less than 2.5%, while generally R.S.D. values were less than 15% for peak area. However, these values could be improved with the addition of an internal standard. Peak area increased linearly on increasing sample introduction time and voltage.

The sensitivity was greater than with the pinched mode, however, there was increased band broadening and the theoretical plates values were lower than that obtained with the pinched mode (<50,000 m^{-1}). However, the gated sample introduction approach proved a more suitable method for determining 2,4-DAPG and MAPG, especially during the early stages of growth, e.g. during the first 8 h, when a low concentration of 2,4-DAPG is released.

3.6. Quantitation of 2,4-DAPG and MAPG in a microbially derived sample

Method development studies involved standards being made up in 50:50% MeOH–borate matrix as the microbially

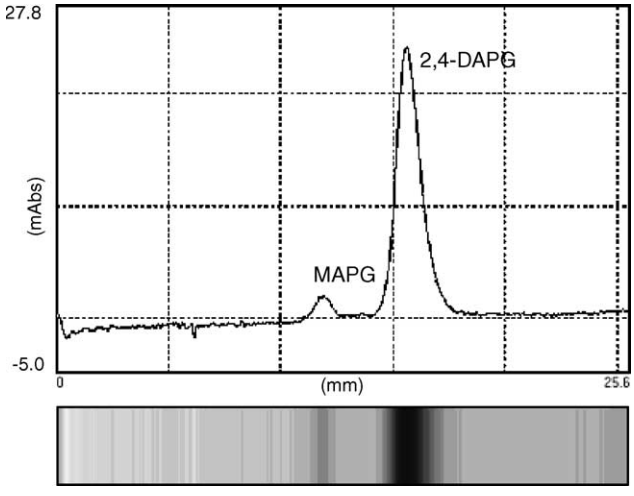

Fig. 3. Chip electropherogram showing a supernatant sample (SPE) taken after 12 h of growth of *P. fluorescens* F113 confirming the presence of MAPG and 2,4-DAPG. Total analysis time 30 s. Separation conditions as in Table 1.

Table 1
Comparison of capillary and microchip electrophoretic separation methods for MAPG and 2,4-DAPG

Parameters investigated	On-chip separation (MCE)	Capillary electrophoretic separation (CE)
Separation platform length	2.5 cm (Quartz microchip)	33 cm (Fused silica capillary)
Separation time	15 s	1.9 min
Limit of detection (MAPG)	<4.0 mg/L	1.2 mg/L
Linear range	10–200 mg/L	10–200 mg/L
Precision (migration time)	<2.5% R.S.D.	<2% R.S.D.
Precision (peak area)	<15% R.S.D.	<5% R.S.D.
Theoretical plates (2,4-DAPG)	2157 (Channel length 2.5 cm)	41400 (Effective length 24.5 cm)

Chip sample introduction settings: SI 1 kV, SO 0 kV, BI 0.34 kV, BO 0.62 kV for 15 s. Separation settings: SI 0.5 kV, SO 0.5 kV, BI 1.7 kV, BO 0 kV for 15 s. Detection at 214 nm, running electrolyte: sodium tetraborate (25 mM, pH 9.3); MAPG, 2,4-DAPG were dissolved in 50:50% MeOH–sodium tetraborate (final concentration 12.5 mM, pH 9.3). CE conditions: running electrolyte 25 mM sodium tetraborate (pH 9.3), voltage 25 kV, 25 °C, hydrodynamic injection (34.5 mbar for 4 s), capillary 50 μm i.d., 375 μm o.d., total length 33 cm, effective length 24.5 cm, detection at 214 nm. MAPG and 2,4-DAPG were dissolved in 100% MeOH.

cultured supernatant samples was eluted using SPE in methanol. From these studies, it was concluded that a minimum of 40% sodium tetraborate borate (pH 9.3) was required for successful sample introduction and 50% (12.5 mM) was chosen as the optimum. Fig. 3 shows the electropherogram obtained in 15 s for a SPE-treated supernatant sample, and the amount of 2,4-DAPG present was 354 mg/L along with 33 mg/L of MAPG, respectively. This was released by the bacterial strain *P. fluorescens* F113 that was cultured as outlined [23]. At this growth stage (12 h), MAPG is produced in low concentrations, however, following 20 and 24 h of growth the metabolite is released at higher concentrations.

3.7. Capillary versus microchip

MAPG and 2,4-DAPG were also separated using a conventional CE system prior to this microchip electrophoresis study [18]. Table 1 highlights the important differences between the two approaches, with microchip electrophoresis greatly reducing separation time. Smaller devices are better at dissipating unwanted joule heating due to increased resistance, and thus lower current flow. Even though the separation channel length of the microchip was 13 times smaller than the capillary length, there was little difference in the electric field strength that was applied. The separation voltage for the microchip method was 0.68 kV/cm, while 0.76 kV/cm was applied for the capillary method. In general, there is less temperature difference inside a microchannel in comparison to a capillary, the internal temperature is more uniform, which gives less convective diffusion and narrower bands. The data in Table 1 shows the capabilities of both systems.

4. Conclusion

It is evident that this microchip format can deliver high-speed separations in seconds, which has many obvious benefits for the pharmaceutical, biomedical and many other sectors. In recent years, this area of research has witnessed significant advancements, which has seen microchip devices being employed for multi-functional lab-on-a-chip including sample preparation, real time separation and detection. The main advantages of a UV detection system over commonly used amperometric systems is ease of use, and for many compounds that are readily UV absorbing, sub-minute quantitation is possible. Laser induced fluorescence (LIF) is frequently incorporated with microchip electrophoresis for detection purposes. Even though fluorescence detection offers greater sensitivity when compared to UV, derivatization with a fluorescent species is often necessary. The amount of antimicrobial metabolites present in supernatant SPE was easily quantitated using linear imaging UV detection. The work shown here may progress to determining phloroglucinol in a plasma matrix on chip; coupled with MS this would represent a powerful analytical tool.

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