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Development of a capillary electrophoretic method for the separation of the macrolide antibiotics, erythromycin, josamycin and oleandomycin

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Abstract

Capillary electrophoresis (CE) provides high separation efficiency and thus is suitable for the analysis of complex mixtures of structurally similar compounds. The versatile nature of CE can be realised by controlling the chemistry of the inner capillary wall, by modifying the electrolyte composition and by altering the physicochemical properties of the analyte. A CE method has been developed for the separation of three macrolide antibiotics, erythromycin, oleandomycin and josamycin. A systematic approach was used to maximise analyte differential electrophoretic mobility by manipulating electrolyte pH, molarity and composition. In addition, some instrumental parameters such as capillary length and diameter and applied voltage were varied. The effect of the sample solvent and on-capillary concentrating techniques such as field amplified sample injection were investigated. Also, the influence of the injection of a water plug on the quantity of sample injected was demonstrated. The macrolides were completely resolved in less than 30 min in a 100 cm×75 μm I.D. fused-silica uncoated capillary with a Z-shaped flow cell of path-length 3 mm. The analysis was performed in a 75 m*M* phosphate buffer (pH 7.5) with 50% (v/v) methanol and an applied voltage of 25 kV was selected to effect the separation.

Keywords: Erythromycin; Oleandomycin; Josamycin

1. Introduction

Macrolide antibiotics are produced by the actinomycetes fungi species streptomyces. Of the macrolide antibiotics currently in clinical use, erythromycin and its derivatives are the most commonly prescribed. The analysis of these compounds has been widely studied using HPLC and LC. Several methods for the separation of erythromycin components in samples of biological origin have been published [1–4]. However, these methods are not suitable for the assay or purity control of erythromycin in bulk and pharmaceutical preparations. Numerous attempts have been made to develop suitable methods for the assay of erythromycin in bulk and dosage forms. Reversed-phase chromatography with either silica-based columns [5–7] or poly(styrene–divinylbenzene) copolymers [8,9] have been used for the analysis of erythromycin. Cachet et

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al. [7] found that slight modifications of the surface of the column packing with aging influenced the quality of the separation. In addition, it was found that interactions between the acidic silanol groups and the amine moiety of the macrolides were reduced in the presence of tetraalkylammonium hydrogen sulphate in the mobile phase. Silanol-deactivated silica-based C_{18} phases were investigated by Paesen et al. [10] for the separation of erythromycin.

Capillary electrophoresis (CE) is an economical, powerful analytical technique, that is capable of providing high efficiency, improved selectivities and rapid analysis times. Due to the different mechanisms of selectivity, CE has the potential to extend or complement the capabilities of other analytical techniques, such as HPLC and LC [11]. Flurer [12] reported the development of a cholate and an acetonitrile system for the separation of six macrolide antibiotics. Baseline resolution of these macrolides was achieved with both systems. Each of these separation systems offered unique selectivity thus providing an additional method of solute identification.

The present paper reports the development of a CE method for the analysis of three macrolide antibiotics; erythromycin, oleandomycin and josamycin. The compounds consist of a macrocyclic aglycone ring to which two sugars are glycosidically linked. As they are sufficiently similar in molecular mass and structure, a separation system that is highly selective is required.

The objective of this work was to study the influence of the experimental variables in CE on the separation of the macrolides. The effects of pH, electrolyte molarity, applied voltage, capillary dimensions, injection solvent and electrolyte additives on the separation were investigated.

2. Experimental

2.1. Chemicals

Erythromycin was purchased from Sigma (St, Louis, MO, USA). Josamycin was donated by Yamanouchi (Tokyo, Japan) and oleandomycin was received from Pfizer (Pietermatrizberg, South Africa). All reagents used were of analytical grade. HPLC grade acetonitrile and methanol was obtained from Burdick and Jackson (Baxter, Muskegon, MI, USA). Analytical grade sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Distilled water was further purified through a Milli-Q System (Millipore, Bedford, MA, USA).

2.2. Instrumental

Capillary electrophoresis was performed on a Prince Instrument (Lauerlabs, Emmem, The Netherlands) coupled to a Butler buffer replenishing device (Lauerlabs). The CE system was equipped with a Model 3390 A Series II integrator (Hewlett-Packard, Avondale, PA, USA). An uncoated fused-silica capillary with a modified Z-shaped flow-through cell was used (LC Packing, San Francisco, CA, USA). The capillary dimensions were as follows: total length, 81.6 cm and effective length, 41.4 cm$\times$75 μm I.D., path-length 3 mm. The capillary was maintained at a constant temperature of approximately 25°C using a thermostated oven. The macrolides were detected by UV absorption at 200 nm and the detector was set at 0.1 absorbance units full scale (a.u.f.s.) with a rise time of 0.3 s.

The mode of injection was electrokinetic, applying 5 kV for a duration of 5 s. Controlled voltage up and down ramping for the injection and electrophoresis was programmed at 6 kV/s. New capillaries were conditioned with 1 *M* NaOH, followed by 0.1 *M* NaOH and then water for 30 min each, using a pressure of 2000 mbars. Prior to use, the capillary was flushed with 0.1 *M* NaOH and water for 10 min each and equilibrated with the background buffer for 15 min. Between consecutive injections the capillary was rinsed with the operating buffer for 3 min.

The sample solution consisted of 1 mg/ml of each macrolide dissolved in methanol. The methanol in the sample solution served as the neutral marker and was used to monitor the electroosmotic flow. The analysis was performed in a phosphate buffer that was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate. The buffer solutions were degassed under aspirator vacuum and filtered through a 0.45-μm membrane filter (Millipore), prior to analysis.

3. Results and discussion

3.1. Optimisation of electrolyte pH

Electrolyte pH is an important parameter that can be manipulated to optimise selectivity in CE. Selectivity is based on the differential electrophoretic mobilities of the analytes. Electrolyte pH induces charge differentiation by altering the extent of dissociation of each individual solute. Thus pH influences the separation by altering the differential electrophoretic mobility of the solutes. Subsequently, it is possible to resolve structurally similar compounds by adjusting the pH [13–17]. The electrolyte pH was examined in the range from 5 to 9 which encompassed the pK_a values of the macrolides (erythromycin, pK_a 8.8; josamycin, pK_a 6.7–7.1; oleandomycin, pK_a 8.7). The acid region was avoided as macrolides are known to be acid-labile [18–20].

Fig. 1 is a graphic representation of the relationship between electrolyte pH and electroosmotic and electrophoretic mobility. The electrophoretic mobility of each compound decreased with increasing pH, while the electroosmotic mobility escalated relatively rapidly. Oleandomycin co-migrated with erythromycin over the entire pH range, as observed from the overlap of their electrophoretic mobilities. Josamycin had a lower mobility than erythromycin and thus a slightly longer migration time. Resolution improved with pH, except that the separation of josamycin from the electroosmotic flow (EOF) marker was not achieved at pH values above 8. The electrophoretic mobility of josamycin approached zero and it co-migrated with the EOF marker.

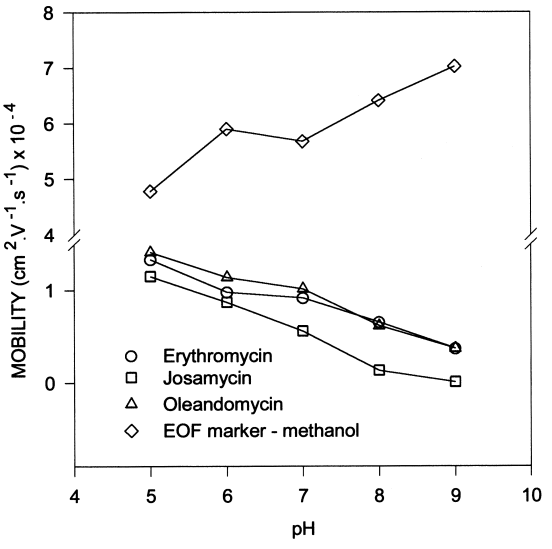

Fig. 1. Variation of electrophoretic and electroosmotic mobility with pH. Conditions: 20 m*M* phosphate buffer (pH 5–9); applied voltage, 15 kV; injection, 5 kV for 5 s; capillary, 81.6 cm (effective length 41.4 cm)×75 μm I.D.; concentration of macrolides, 1 mg/ml in methanol.

In addition to electrolyte concentration and viscosity, the electrophoretic mobility is dependent on the charge and size of the ion [15]. The ionic equilibrium state of the analyte is largely dependent on the pH, especially when it is within 2 pH units of the pK_a value of the analytes [13,15,21]. It is possible that the observed decrease in the electrophoretic mobility of the macrolides, with increasing pH, was due to the decrease in the fraction of protonated species. In principle, resolution can be improved by increasing the difference in the electrophoretic mobility of the analytes and decreasing the EOF [22]. The decrease in the differential electrophoretic mobilities of the macrolides and the increase in the EOF at high pH values resulted in the impairment of resolution. Therefore, a pH value of 7.5 was selected as the optimal pH for the separation of the macrolides.

3.2. Effect of applied voltage

Ions in the electrophoretic medium migrate as a result of the voltage applied across the capillary. Fig. 2 shows the relationship between the electroosmotic and electrophoretic mobility and applied voltage. Solute velocity was linearly proportional to the applied voltage as the Joule heat generated was adequately dissipated. High voltages reduced the analysis time and sharpened the peaks by minimising zone broadening. Theoretically, resolution and efficiency are directly proportional to the applied voltage [23]. However, the inability of the system to remove the excess Joule heat generated at high voltages, results in peak broadening and a decrease in efficiency and resolution [24]. Resolution and efficiency approached a maximum as the applied voltage was increased (20 kV) and then dropped as the voltage was further increased. An applied voltage

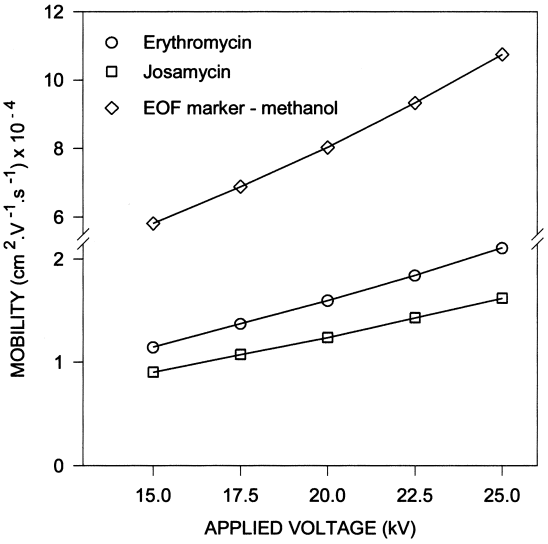

Fig. 2. Variation of electrophoretic and electroosmotic mobility with applied voltage. Conditions: 20 m*M* phosphate buffer (pH 7.5); applied voltage, 15–25 kV; other conditions as for Fig. 1.

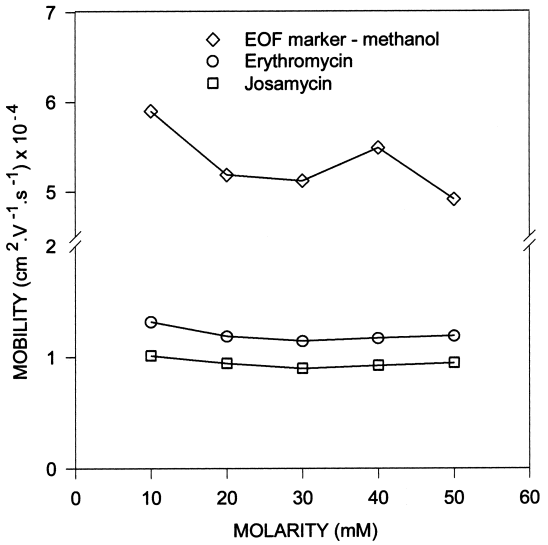

Fig. 3. Variation of electrophoretic and electroosmotic mobility with electrolyte molarity. Conditions: 10–50 m*M* phosphate buffer (pH 7.5); applied voltage, 17.5 kV; other conditions as in Fig. 1.

of 17.5 kV was selected for the analysis. High efficiency and short analysis times, without the generation of detrimental amounts of heat were achieved at this voltage.

3.3. Effect of electrolyte molarity

The electrolyte molarity influences the separation in several ways and can be utilised to enhance the efficiency, selectivity and resolution [15,24,25]. The EOF, current produced in the capillary and solute adsorption effects are all affected when the buffer molarity is altered. Thus, the optimal electrolyte molarity has to be determined empirically. In this study, phosphate buffers (pH 7.5) of molarity ranging from 10 m*M* to 50 m*M* were prepared. Fig. 3 illustrates the effect of electrolyte molarity on the electroosmotic and electrophoretic mobility. The reduction in the EOF with increasing molarity is due to the contraction of the electrical double layer and reduction of the surface charge/unit area [15,26]. The improvement in resolution and lengthened analysis time observed with electrolytes of high molarity may be attributed to the reduction of the EOF since the differential mobilities of the compounds remained invariant over the concentration range. A concentration of 20 m*M* was selected in terms of analysis time, current generated and resolution.

3.4. Effect of capillary dimensions

The capillary diameter is a critical parameter as it regulates the quantity of Joule heat that is generated and the magnitude of the thermal gradient across the capillary [27,28]. In addition, the capillary diameter determines the detection sensitivity [29,30]. The effect of the capillary diameter on the peak height is illustrated in Fig. 4a. The improvement in detection sensitivity in wide bore capillaries resulted from the increase in path-length, volume of the capillary employed as the on-capillary detection cell and amount of sample injected onto the capillary. However, the use of wide bore capillaries is limited by the inability of the capillary to remove the Joule heat generated and the development of a parabolic flow profile. These effects result in peak broadening and reduce system efficiency. Consequently, a compromise between the detector sensitivity and the level of Joule heat produced is necessary in selecting the capillary diameter for the analysis. A diameter of 75 μm was found to be appropriate for the analysis in

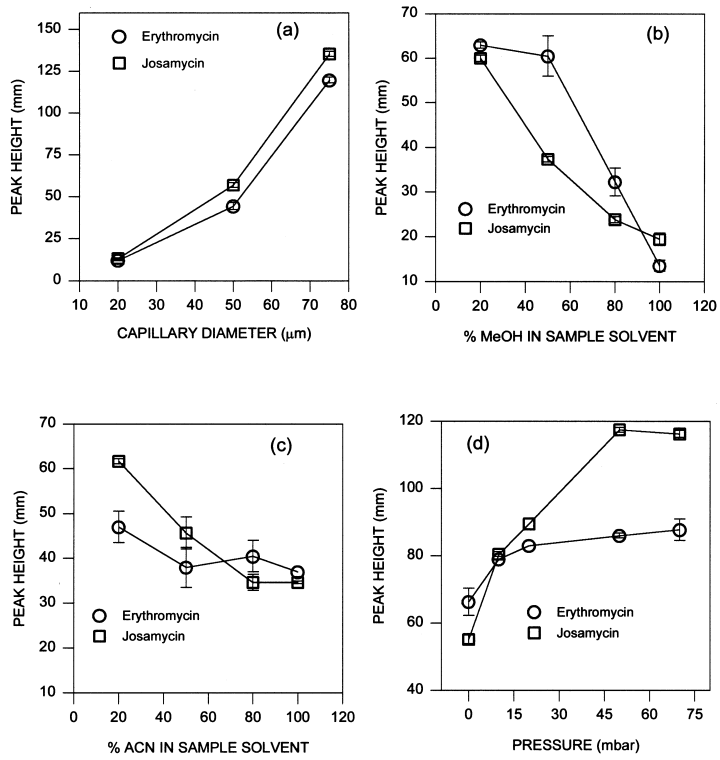

Fig. 4. Peak height as a function of (a) capillary diameter, (b) percentage methanol in sample solvent, (c) percentage acetonitrile in sample solvent, (d) length of water plug expressed in pressure units. Conditions: total capillary length, 114.5 cm; effective capillary length, 75 cm; 20 m*M* phosphate buffer (pH 7.5); applied voltage, 17.5 kV; capillary diameter, 75 μm.

terms of detection sensitivity and Joule heat generation.

In CE, standard capillary lengths employed range from 50 to 100 cm. The capillary length determines the magnitude of the electric field and the quantity of Joule heat generated. The analysis time was found to increase three-fold with the doubling of the capillary length. Resolution improved with increasing length, however, additional peak broadening was observed with effective lengths greater than 100 cm which limited the use of long capillaries. Optimal resolution and short analysis time were apparent with a capillary of total length 100 cm and effective length 75 cm.

3.5. Injection solvent

Improvement in detection sensitivity to compensate for the low sample capacity of the capillary remains a challenge in CE. The on-capillary concentrating technique that is performed by electrokinetically injecting a sample prepared in a solvent of lower conductivity than the electrolyte is termed field amplified sample injection (FASI) [31,32]. This is a desirable technique in that it lowers the detection limits by facilitating on-capillary concentration and by allowing the introduction of larger amounts of ions into the capillary. The electric field at the injection point is amplified and subsequently the peak height and area should effectively increase with injection solvents of lower conductivity.

The low solubilities of the macrolides in aqueous solutions necessitated the inclusion of 20% (v/v) organic solvent in the sample diluent, to prevent precipitation. The sample solvents investigated were methanol–water and acetonitrile–water, in concentrations ranging from 20 to 100% (v/v). A maximum detection sensitivity was obtained with the inclusion of 20% (v/v) methanol or acetonitrile in the sample solvent (Fig. 4b and c). Both peak height and area

decreased with the incorporation of organic solvents of concentration greater than 20% (v/v) in the sample solvent. The difference in conductivity between the sample plug and electrolyte solution resulted in the superimposition of a parabolic flow profile onto the plug flow profile. Consequently, peak broadening became apparent and peak height decreased.

The quantity of sample injected onto the capillary when applying the electrokinetic mode of injection is dependent on the solute mobility, EOF, solute concentration and capillary dimensions [33]. The reduction in the peak area with incremental percentages of methanol in the sample solvent is due to less sample being loaded onto the capillary under the same injection conditions. This possibly occurs as a result of the decrease in the EOF and electrophoretic mobility of the macrolides. The degree to which organic solvents affect the peak height and area is dependent on their chemical properties. Acetonitrile has a negligible effect on the viscosity and dielectric constant of the sample diluent in comparison to methanol [17]. Subsequently, the EOF and electrophoretic mobility of the macrolides were altered minimally in the presence of acetonitrile. Methanol, in contrast, caused a greater decrease in the EOF and electrophoretic mobility as it reduces the dielectric constant and increases the viscosity of the medium significantly.

The pK_a values of the macrolides in aqueous–organic solvents are significantly different to those reported in aqueous solutions. In addition, organic solvents differ in their ability to solvate ions. Consequently, the degree of ionisation of the analytes and the hydrodynamic radius of the ions and thus their electrophoretic mobility were altered. Furthermore, the ionic charge of the analytes was reduced in the presence of organic solvents, as the lowered dielectric constant of the medium favoured neutralisation. The decrease in the fraction of protonated species resulted in a reduction in the electrophoretic mobility of the macrolides. As a result, fewer ions were injected onto the capillary in the mixed aqueous–organic systems.

The significant decrease in peak height (approximately 70% v/v), observed with the inclusion of increasing concentrations of methanol in the sample solvent (Fig. 4b), possibly arose from the higher viscosity of the sample diluent. This induced a laminar flow, which resulted in pronounced peak broadening. Detection sensitivity was optimal with the sample diluent methanol–water (20:80, v/v) as observed from the peak height.

3.6. Effect of water plug

The effect of the length of the water plug on peak height is illustrated in Fig. 4d. Water plugs of various lengths were hydrodynamically injected into the capillary, prior to the sample, by modifying the injection pressure between 10 and 70 mbar for 1 s. A 40% decrease in peak height for both erythromycin and josamycin was observed with increasing lengths of water plugs. The increase in peak height was attributed to the void region created by the water plug into which the sample can be introduced [31,32]. Subsequently, a greater number of ions were injected into the capillary as shown by the increase in peak height. It is feasible to improve the limit of detection in CE by employing FASI with or without a water plug.

3.7. Electrolyte additives

The addition of organic solvents to the electrolyte solution has been shown to improve analyte solubility, detector sensitivity [34] and resolution [14,15,34,35] and control of the EOF [14,15,35,36]. Organic modifiers facilitate the separation of compounds that exhibit very similar electrophoretic mobilities in aqueous electrolytes as observed with the resolution of polyaromatic hydrocarbons [36], positional isomers [37] and isotopically substituted compounds [21]. They change the charge–mass ratio and thus electrophoretic mobility of the solutes and influence the EOF.

Three organic solvents (acetonitrile, methanol and ethanol) were examined at various concentrations. Figs. 5–7 illustrate the improvement in resolution with the incorporation of organic solvents at increasing concentrations in the electrolyte. Resolution was enhanced in the presence of methanol and additional peaks were resolved with concentrations of methanol in excess of 30% (v/v). These peaks are unknown compounds. The migration time escalated significantly with increasing proportions of methanol.

Optimal resolution was achieved with the inclusion of 50% (v/v) methanol in the electrolyte. On replacing methanol with ethanol, optimal separation of the macrolides was achieved with the inclusion of 30% (v/v) ethanol. However, the analysis time increased four-fold and distortion of peak shape with peak height deterioration and peak broadening was observed with the addition of 50% ethanol. Using acetonitrile as the organic modifier, increasing proportions of this solvent in the electrolyte improved resolution, with 30% (v/v) yielding optimal separation. Co-migration of josamycin with the EOF marker was apparent with 60% (v/v) acetonitrile in the electrolyte. Peak shape was not adversely affected at high concentrations of acetonitrile as the EOF was not substantially reduced.

The improvement in resolution in the presence of organic solvents is due to several factors. Organic solvents increase viscosity and reduce the dielectric constant of the electrolyte and decrease the zeta potential [14,15,17,37]. Furthermore, the ionic strength and pH of the electrolyte are altered in the presence of organic modifiers [35]. Subsequently, the electroosmotic and electrophoretic mobilities of the analytes are reduced.

Acetonitrile behaves as an inert solvent in combination with water [17] and thus nominally alters the EOF. Acetonitrile has minimal effect on the viscosity and dielectric constant of the electrolyte [17]. As a result, more of this solvent was required to improve resolution (60% (v/v) of acetonitrile) (Fig. 5). Protic solvents, such as methanol and ethanol, are more efficient in increasing the viscosity and reducing the dielectric constant of the electrolyte [17]. Therefore, the decrease in the EOF was substantially greater than in the presence of acetonitrile. As a result, less of these solvents were required to improve the resolution of the macrolides (50% (v/v) methanol (Fig. 6) and 30% (v/v) ethanol) (Fig. 7). Ethanol increased the electrolyte viscosity and reduced the dielectric constant to a greater extent than methanol thus affecting the mobility and migration time significantly. Optimal resolution of the macrolides was achieved in the presence of methanol with respect to analysis time and peak shape.

In addition to altering the EOF, organic solvents affect the electrophoretic mobilities of the analytes (as discussed above). This is apparent from the shift

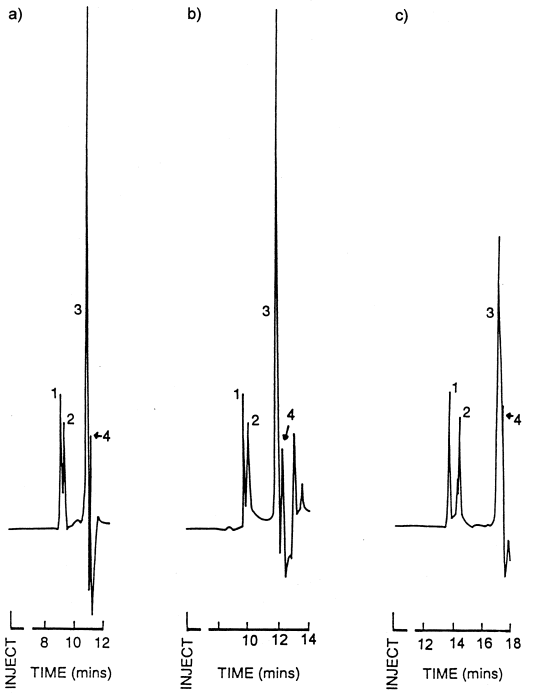

Fig. 5. Electropherogram of the mixture of the macrolides, erythromycin (1), oleandomycin (2), josamycin (3), analysed in electrolyte containing various proportions of acetonitrile. Conditions: 75 m*M* phosphate buffer (pH 7.5); injection; 50 mbar for 5 s; concentration of macrolides; 0.5 mg/ml in methanol–water (10:80, v/v); EOF marker (4). (a) 25% acetonitrile, applied voltage 15 kV; (b) 30% acetonitrile, applied voltage 15 kV; (c) 60% acetonitrile, applied voltage 15 kV.

in mobility and relative migration order of the analytes, when organic modifiers are included in the electrolyte. Selectivity is thus augmented in mixed aqueous–organic systems.

3.8. Optimised separation conditions

The optimal conditions for the separation of the macrolides as determined from the examination of the various experimental variables were found to be: 75 m*M* phosphate buffer (pH 7.5), 50% (v/v) methanol, applied voltage 25 kV, total capillary length, 114.5 cm, effective capillary length, 75 cm, I.D. 75 μm, pathlength 3 mm. The CE method for the analysis of the macrolide antibiotics is simple, requires minimal sample and electrolyte preparation

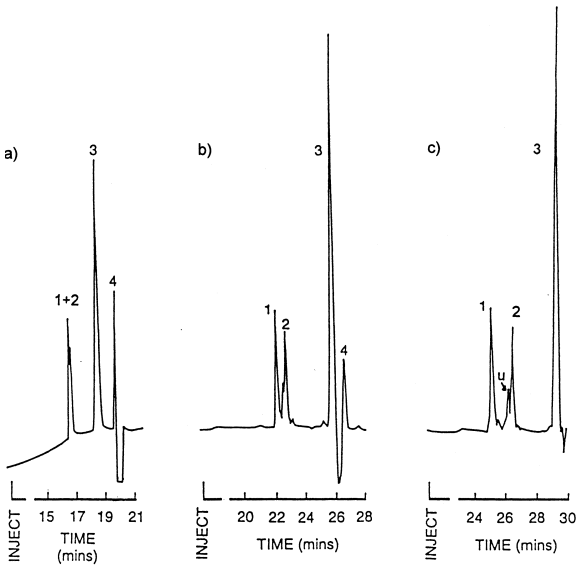

Fig. 6. Electropherogram of the mixture of the macrolides, erythromycin (1), oleandomycin (2), josamycin (3), analysed in electrolyte containing various proportions of methanol. Conditions: 75 m*M* phosphate buffer (pH 7.5); injection, 50 mbar for 5 s; concentration of macrolides, 0.5 mg/ml in methanol–water (10:80, v/v); EOF marker (4); Unknown (U). (a) 0% methanol, applied voltage 15 kV, (b) 30% methanol, applied voltage 20 kV, (c) 50% methanol, applied voltage 25 kV.

and utilises small quantities of sample and organic solvent.

4. Conclusions

The influence of the experimental parameters in CE on the resolution of the macrolides was systematically examined. Numerous factors need to be taken into consideration when selecting the optimal conditions for the separation. Of significance, is the influence of the experimental parameters on the production of Joule heat. Excess Joule heat is detrimental to the quality of the separation and should be minimised. In CE several factors have a combined effect. High molarity electrolytes and high applied voltages can increase the heat generated within the capillary independently. If both factors are increased simultaneously, then the increase in the amount of Joule heat will be cumulative and may impair separation performance significantly. Therefore, the effect of each variable on the separation must be considered in conjunction with other variables.

It has been demonstrated that the above system is capable of resolving structurally similar compounds.

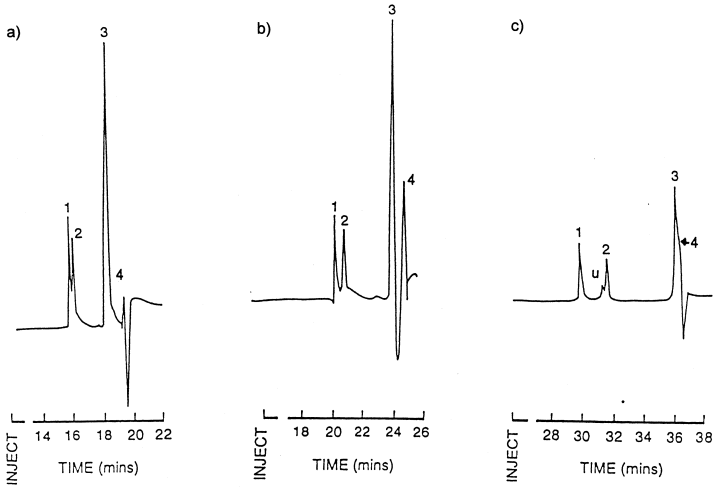

Fig. 7. Electropherogram of the mixture of the macrolides, erythromycin (1), oleandomycin (2), josamycin (3), analysed in electrolyte containing various proportions of ethanol. Conditions: as in Fig. 5. EOF marker (4); Unknown (U). (a) 10% ethanol, applied voltage 20 kV, (b) 30% ethanol, applied voltage 25 kV, (c) 50% ethanol, applied voltage 30 kV.

This implies that the separation system has the potential to resolve erythromycin from its related substances and degradation products. The differential electrophoretic mobilities of the macrolides were maximised by systematically modifying several parameters. The effect of each parameter on the electrophoretic and electroosmotic mobility and resolution was demonstrated. The inclusion of the organic modifiers in the electrolyte appeared to have altered the electrophoretic mobilities substantially, thereby improving resolution.

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