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Determination of erythromycin and related substances by capillary electrophoresis

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Abstract

Current compendial methods of assay for the analysis of erythromycin and its related substances involve the use of microbiological techniques. These techniques are non-selective and tedious, thus there is a need for the development of highly specific, quantitative analytical methods. Erythromycin was analysed in a 50 m*M* phosphate buffer (pH 7.5) and run at an applied voltage of 20 kV. Detection sensitivity was enhanced by using a wavelength of 200 nm and selecting an injection solvent of lower conductivity than the electrolyte: acetonitrile–water (20:80, v/v). In order to facilitate the separation of erythromycin and its related substances, the organic solvent ethanol (35%, v/v) was incorporated into a modified 150 m*M* phosphate buffer (pH 7.5) and run at an applied voltage of 30 kV. Resolution of all the compounds was achieved in approximately 45 min. The methods described are accurate and precise and thus suitable for the quantitative determination of erythromycin and the related substances, erythromycin C, anhydroerythromycin and N-demethylerythromycin A. © 1997 Elsevier Science B.V.

Keywords: Erythromycin; Erythromycin C; Anhydroerythromycin; N-Demethylerythromycin A

1. Introduction

Capillary electrophoresis (CE) is rapidly gaining acceptance as an alternative and complementary analytical technique to HPLC for the analysis of pharmaceutical raw materials and dosage forms [1–7]. The many benefits afforded by this technique and the availability of fully automated PC-controlled CE systems have contributed significantly to the increasing attention being given to this method of analysis. High separation efficiency, large separatory capacity, improved selectivity, low operational costs, speed of analysis, simplicity and minimal method development are some of the principle advantages of CE [8,9].

Several reports have been published, focusing on the quantitative aspects of CE [10,11]. Altria and Rudd [12] have established the specific requirements for the validation of CE methods. As no official guidelines are available for the validation of CE methods, the validation criteria employed are similar to those used in evaluating the performance of chromatographic methods [5,7,12].

Currently the US Pharmacopeia (USP) [13] and the British Pharmacopeia (BP) [14] use microbiological methods for the assay of erythromycin. Microbiological assays are limited as they lack specificity and are unable to differentiate between the active and inactive moieties. Furthermore, these

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assays are not stability-indicating. The BP includes a thin-layer chromatography (TLC) method for related substances, but does not make provision for the identity of these related components. Recently, the European Pharmacopeia (EC) [15] adopted an HPLC method (Paesen et al. [16]) capable of separating erythromycin E from the parent compound. However, the method is complex and does not allow separation of the other related substances within a reasonable time. Nars and Tschappler [17] have recently described an improved HPLC method which is capable of assaying erythromycin and most related substances commonly found in commercial samples.

Several TLC methods [18,19] have been developed, but these are not suited to quantitative work. Liquid chromatography (LC) methods [20–25] have also been developed for the assay of erythromycin. A column-switching technique has also been used to separate erythromycin from all its know impurities [26]. However, this method is complex and tedious to perform.

In the present study, an assay method for erythromycin has been developed and validated. Slight modifications of the running conditions, by incorporating 35% (v/v) ethanol in a buffer of increased molarity and using higher run voltages have further enabled the separation of erythromycin and its related substances. Method validation included measures of precision, linearity, accuracy, sensitivity and stability.

2. Experimental

2.1. Chemicals

Erythromycin was purchased from the USP Convention (Rockville, MD, USA) and josamycin was donated by Yamanouchi (Tokyo, Japan). Erythromycin A, B, C and N-demethylerythromycin A were obtained from the European Pharmacopeia (Strasbourgh, Cedex 1, France). Abbott (North Chicago, IL, USA) donated erythromycin enol ether and anhydroerythromycin and phenylpropanolamine was obtained from Lennon (Port Elizabeth, South Africa). HPLC grade acetonitrile and ethanol were purchased from Burdick and Jackson (Baxter, Muskegon, MI, USA). Analytical grade sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Distilled water was further purified through a Milli-Q System (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Capillary electrophoresis was performed on a Prince Instrument (Lauerlabs, Emmen, 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). Analysis was performed in an uncoated fused-silica capillary of total length 144.5 cm and effective length 75 cm×50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). The capillary was thermostated at approximately 30°C. Detection was by UV absorption at 200 nm and the detector was set at 0.005 absorbance units full scale (a.u.f.s.) with a rise time of 0.3 s.

Samples were injected using the hydrodynamic mode of injection, applying 50 mbar pressure for 5 s. Controlled voltage up and down ramping for the run voltage 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 mbar. 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.

3. Results and discussion

3.1. Assay of erythromycin

Electrolyte pH is an important parameter that can be manipulated to optimise selectivity in CE as it enhances mobility differences between the solutes and affects the electroosmotic flow (EOF). The electrolyte pH was examined in the range from 5 to 9 and a pH of 7.5 was selected as the optimal pH for the analysis of erythromycin.

An applied voltage of 20 kV was used to effect the separation. Short analysis times and high efficiency were achieved at this voltage. Detrimental amounts

of Joule heat were not generated at this voltage when employing a 50 m*M* phosphate buffer.

Sensitive detection of erythromycin presents a challenge as it lacks a significant chromophore. Therefore, a low UV detection wavelength (200 nm) was selected at which most solutes have enhanced absorptivity, thereby compensating for the relative insensitivity of CE. Detection sensitivity can be further improved by using on-capillary concentrating techniques such as stacking and field amplified sample injection (FASI). A sample solvent of lower conductivity than the electrolyte (acetonitrile–water 20:80, v/v) was selected in which optimal detection sensitivity was achieved. This sample solvent facilitated stacking and resulted in concentration enrichment of the analyte zone prior to separation without compromising resolution.

An electrolyte rinse cycle was included in the experimental sequence prior to each injection to improve migration time and peak response precision. Two blank injections of sample solvent were performed at the start of each experimental set to equilibrate the silica surface of the capillary.

3.2. Method validation for the assay of erythromycin

3.2.1. Precision

Replicate analyses (ten consecutive injections) of a standard solution of erythromycin, at a concentration of 0.8 mg/ml, were performed and resulted in precision data having a R.S.D. value of 0.10% for migration time, 1.71% for normalised peak area and 0.72% for peak height. Josamycin (0.25 mg/ml)was selected as the internal standard (I.S.) to improve peak area and migration time precision. Variability was reduced in the presence of the I.S. as observed from the R.S.D. values of the relative migration time (0.03%), relative normalised peak area (1.4%) and relative peak height (0.83%). Migration time precision in CE is typically within the range of 0.5–1.0% R.S.D. [7,27,28]. Precision levels of 0.5–2.0% R.S.D. for peak areas are generally achieved in CE when using automated instrumentation [27,28]. Typically, R.S.D. values of below 1% are achieved by employing an I.S. as fluctuations in migration time and injection volume are thereby compensated [29].

Intermediate precision was assessed on different days and R.S.D. values of less than 0.05% were obtained for relative migration time, 2.33% for relative normalised peak area and 1.33% for relative peak height.

3.2.2. Linearity

For the establishment of linearity, six standards of varying concentration of erythromycin were analysed in triplicate on each of three days. Detector linearity for peak area ratios was assessed over the range 0.1–1.4 mg/ml (equivalent to 10–140% of the nominal assay concentration). Acceptable linearity was demonstrated with an average correlation coefficient of 0.994.

3.2.3. Accuracy

Accuracy was determined concurrently with linearity on each of three days. Three solutions of erythromycin covering the concentration range (0.1–1.4 mg/ml) were analysed in triplicate and the erythromycin concentration in each sample was calculated using the appropriate regression equation. The results were in close agreement with the actual concentration of erythromycin in the sample solution. The mean percentage bias ranged from 0.8 to 4.76% and the mean percentage recovery from 99 to 105%.

3.2.4. Sensitivity

The limit of detection (LOD) was determined to be 0.03 mg/ml (equivalent to 3% of the nominal concentration), based on a signal-to-noise ratio of three. This is the lowest concentration of analyte that can be detected above the baseline noise. The minimum level at which erythromycin can be quantitated with acceptable precision and accuracy was determined to be 0.04 mg/ml (equivalent to 4% of the nominal concentration) where the % R.S.D. was below 10%. Acceptable R.S.D. values ranging from 1.09 to 3.90% for relative normalised peak area precision were obtained on performing ten replicate injections at the limit of quantitation (LOQ).

3.2.5. Stability

A stock solution of erythromycin was prepared and stored in the refrigerator at 4°C and on the bench top at 22°C. On each of three days the samples were analysed. No additional peaks were observed and the

peak area ratios remained consistent throughout the stability study. Erythromycin was found to be stable in acetonitrile as demonstrated by Terespolsky and Kanfer [30] and the storage conditions were not found to impair stability.

The CE method developed for the quantitation of erythromycin is rapid, precise, accurate and stability-indicating. It can be used routinely for the determination of erythromycin in bulk drug as an alternative and complement to existing HPLC methods.

3.3. Method development for the analysis of related substances

The running conditions used for the erythromycin assay were slightly modified to effect the resolution of the related substances. Ethanol (35% v/v) was incorporated into a modified 150 m*M* phosphate buffer (pH 7.5) of increased molarity and run at 30 kV. Structures of the related substances are illustrated in Fig. 1. The high peak efficiencies that are obtained in CE are conducive to discriminating between structurally similar compounds.

Injection was performed hydrodynamically, applying 100 mbar for 5 s. Several parameters were manipulated to achieve resolution of erythromycin and its related substances by reducing the EOF and augmenting the differential electrophoretic mobilities of the compounds present in the mixture.

The electrolyte molarity was examined in the range 50 to 190 m*M*. The EOF is reduced in electrolytes of high molarity. However, the current level and amount of Joule heat generated is substantially greater, thus limiting the applied voltages that can be used to effect the separation. Different organic solvents (methanol and ethanol) at concentrations of between 25 and 50% (v/v) were incorporated into the electrolyte. Organic modifiers enhance the differences in electrophoretic mobility of the analytes and thus improve the separation selectivity. In addition, organic solvents reduce the current level and thus permit the use of high voltages without generating excessive amounts of Joule heat. However, the electrolyte molarity and percentage of organic solvent incorporated into the electrolyte needs to be carefully selected to prevent the precipitation of the buffer salts in high molarity electrolytes with large concentrations of organic solvents. Improved resolution was achieved in the presence of ethanol, compared to methanol, possibly due to the greater decrease in the EOF. Ethanol possesses a higher viscosity and lower dielectric constant than methanol [31].

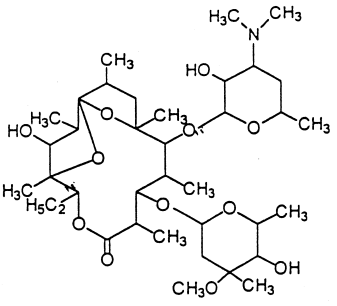
Electroosmotic modifiers such as hydroxyethylcellulose (HEC) and poly(vinyl alcohol) (PVOH) were incorporated into the buffer in various proportions (0.05 to 0.1% w/v), to reduce the EOF and subsequently improve the resolution between the analytes. However, there was no apparent improvement in resolution under these conditions. Coated capillaries, CElect P150 and CElect H150 of total length 100 cm and effective length 65 cm×75 μm I.D. (Supelco, Bellefonte, PA, USA) were also employed in an attempt to improve the resolution between the components. Although the EOF was slightly reduced in the coated capillaries, resolution was not enhanced using these capillaries.

The optimal conditions for the resolution of erythromycin and its related substances were determined: uncoated fused-silica capillary total length 132 cm, effective length 75 cm×75 μm I.D., 150 m*M* phosphate buffer (pH 7.5), 35% (v/v) EtOH. Fig. 2 represents an electropherogram showing the resolution of erythromycin and its related substances.

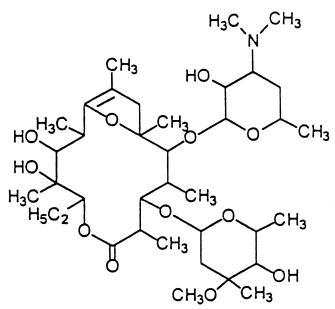
3.4. Method validation for erythromycin related substances

The determination of drug-related impurities is currently considered to be a primary goal for CE in pharmaceutical analysis [5–7,12,32,33]. Capillary electrophoresis can be used to verify HPLC impurity data. Good agreement between these two techniques confirms analytical results obtained as the separation mechanisms on which these techniques are based are different. A comprehensive review on the analysis of drug-related impurities has been published by Altria [32,33].

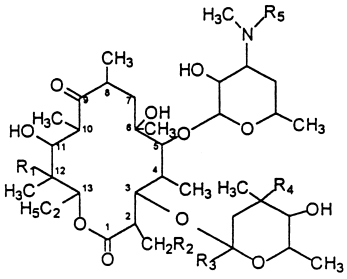
The assay of related substances in bulk samples presents a formidable challenge since the parent compound invariably interferes with the determination of the minor components. Although all the related substances (EB, EC, EEE, AE and NDEA) when present in equal amounts were successfully resolved (Fig. 2), only EC, AE and NDEA could be



Anhydroerythromycin



Erythromycin enol ether



Erythromycin

	R_1	R_2	R_3	R_4	R_5
Erythromycin A	OH	H	H	OCH_3	CH_3
Erythromycin B	H	H	H	OCH_3	CH_3
Erythromycin C	OH	H	H	OH	CH_3
Erythromycin D	H	H	H	OH	CH_3
Erythromycin E	OH	-O-		OCH_3	CH_3
Erythromycin F	OH	OH	H	OCH_3	CH_3
N-demethylerythromycin A	OH	H	H	OCH_3	H

Fig. 1. Chemical structures of erythromycin and its related substances and impurities.

quantitatively determined in the presence of large amounts of EA.

3.5. Precision

Phenylpropanolamine (0.02 mg/ml) was employed as the I.S. to improve quantitative analysis of the CE method. Several macrolides were investigated for possible use as internal standards but they all co-migrated with one or other of the components in the mixture. The R.S.D. values for eleven consecutive injections of a standard stock solution of EA (5 mg/ml) and EC, AE and NDEA (0.25 mg/ml) are tabulated in Table 1. Typically R.S.D. values of below 10% are acceptable for impurities at low levels [6,7,27,32].

Intermediate precision was assessed by performing a number of consecutive injections of the sample solution of EA (5 mg/ml) and EC, AE and NDEA (0.25 mg/ml) on each of 3 days. Table 2 illustrates the R.S.D. values for each compound in the mixture. Precision values of below 10% were consistently

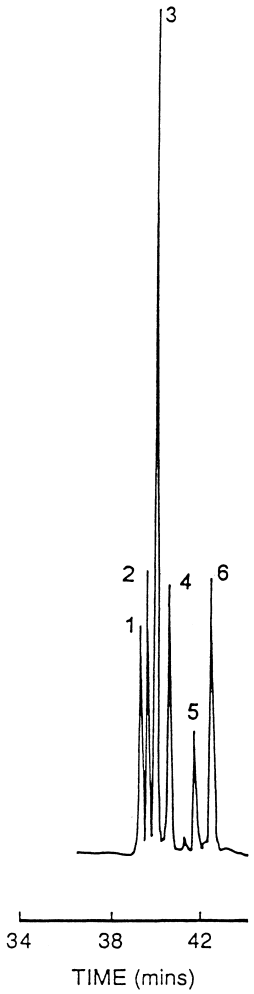


Fig. 2. Electropherogram of erythromycin and its related substances. Conditions: 150 m*M* phosphate buffer (pH 7.5), 35% (v/v) EtOH; injection, 100 mbar for 5 s; applied voltage, 30 kV. Sample concentration 0.5 mg/ml in acetonitrile–water (20:80, v/v). Erythromycin B (1), erythromycin A (2), erythromycin enol ether (3), erythromycin C (4), anhydroerythromycin (5), N-demethylerythromycin (6).

achieved. Several minor factors such as minimising siphoning effects and evaporation, regular washing of the capillary to ensure a constant EOF, electrolyte replenishment and voltage ramping were taken into consideration to reduce the level of variation.

3.6. Linearity

Detector linearity was assessed on three days by analysing five standards solutions containing EA at a constant concentration of 5 mg/ml and EC, AE and NDEA at concentrations ranging from 10 to 200% of the nominal assay concentration (0.5 mg/ml). Calibration lines for each day were constructed by plotting the relative normalised peak area of each compound to that of the I.S. as a function of the analyte concentration. A regression line obtained by the method of least squares was calculated to evaluate the results. Acceptable detector linearity was obtained within the range 10–200% with correlation coefficients of 0.995 for erythromycin C, 0.997 for anhydroerythromycin and 0.995 for N-demethylerythromycin for the relative normalised peak areas respectively. The intercept values for the relative normalised peak area were close to zero.

3.7. Accuracy

The accuracy of the method for each of the related substances in the presence of large quantities of the parent (erythromycin base 5 mg/ml) was assessed, by spiking a solution of erythromycin base in acetonitrile–water (20:80, v/v) with 0, 20, 100 and 200% (v/v) of a mixture containing EC, AE and NDEA (0.5 mg/ml of each impurity). Accuracy was determined concurrently with linearity by injecting each solution in triplicate (Table 3). The mean concentrations found on spiking the parent com-

Table 1
Inter-assay precision for erythromycin C (EC), anhydroerythromycin (AE) and N-demethylerythromycin (NDEA) (% R.S.D.)

Compound	Migration time	Relative migration time	Normalised peak area	Relative normalised peak area	Peak height	Relative peak height
EC	0.76	0.31	2.70	1.77	1.40	1.40
AE	0.82	0.47	2.25	3.72	3.05	3.18
NDEA	0.61	0.32	1.05	2.55	2.32	2.87

$n=10$.

Table 2
Intermediate precision for erythromycin C (EC), anhydroerythromycin (AE) and N-demethylerythromycin (NDEA), (% R.S.D.)

Compound	Migration time	Relative migration time	Normalised peak area	Relative normalised peak area	Peak height	Relative peak height
EC	3.05	1.16	4.45	3.99	2.57	5.47
AE	4.91	1.23	7.43	2.46	3.85	5.39
NDEA	2.98	1.20	3.58	2.80	3.34	5.02

$n=25$

pound with various concentrations of impurities were very close to the actual concentrations present in the spiking solution for all three compounds tested. Improved accuracy was generally observed at higher sample concentrations. Typically, a recovery limit of between 90–110% of the theoretical value is acceptable as was observed in this study. The accuracy data for EC, AE and NDEA in this study were all within the acceptable range.

3.8. Sensitivity

Although UV detection is utilised in both CE and HPLC procedures, the narrow diameter of the capillary and consequently extremely short pathlength used as the flow-through cell in CE results in diminished sensitivity. Concentrations at which the % R.S.D. values were less than 20% and where the peaks were visible above the baseline (>2.0 mm), were reported as the LOD. The LOQ was determined as the concentration at which % R.S.D. values of less than 15% were obtained. The LOQ values for erythromycin related substances are presented in Table 4. At a detection wavelength of 200 nm, EA, EB and EC have comparable UV absorptivities, while AE and NDEA possess significantly lower UV absorptivities. The specific absorbance of EEE is more than ten times greater than that for the other related substances. Consequently, the LOD and LOQ values are better than those for AE and NDEA.

3.9. Stability

A stock solution containing EA (5 mg/ml) and EC, AE and NDEA (0.25 mg/ml) was stored in the refrigerator at 4°C and on the bench top at 22°C for a period of 3 weeks. On day 1 and on each of three days over 21 days, stock solutions stored in the

Table 3
Accuracy data - erythromycin C (EC), anhydroerythromycin (AE) and N-demethylerythromycin (NDEA)

	Spiking level (%)	Actual concentration (mg/ml)	Mean calculated concentration		Mean bias (%)	R.S.D. (%)	Mean recovery (%)	R.S.D. (%)
			(mg/ml)	R.S.D. (%)				
Erythromycin C	20	0.049	0.051	0.21	3.92	0.19	104.1	0.23
$y=2.11x-0.01$	100	0.245	0.240	0.15	−2.08	0.14	98.0	0.12
	200	0.490	0.490	0.11	0.00	0.08	100	0.08
Anhydroerythromycin	20	0.050	0.052	0.25	3.85	0.25	104.0	0.24
$y=0.76x-0.01$	100	0.250	0.250	0.14	0.00	0.13	100.0	0.11
	200	0.500	0.510	0.09	1.96	0.07	102.0	0.07
N-demethylerythromycin	20	0.052	0.051	0.23	−1.96	0.22	98.0	0.23
$y=1.36x-0.02$	100	0.260	0.267	0.15	2.62	0.11	102.7	0.12
	200	0.520	0.518	0.08	−0.39	0.06	99.62	0.07

$n=3$

Table 4
Limit of quantitation for erythromycin C (EC), anhydroerythromycin (AE) and N-demethylerythromycin (NDEA)

Compound	Concentration (mg/ml)	Nominal concentration (%)	Relative normalised peak area R.S.D. (%)	Peak height R.S.D. (%)
EC	0.025	10	4.86	6.90
AE	0.0375	15	6.83	8.60
NDEA	0.0375	15	5.05	8.00

refrigerator and on the bench top were analysed. No additional peaks were detected and the peak area ratios were consistent over this time period.

4. Conclusions

Methods have been developed and successfully validated for the assay of erythromycin and the related substances EC, AE and NDEA. The inability of this assay to resolve EA from EB and EEE when large concentrations of EA are present, precludes its use for the quantitative determination of EB and EEE. The methods developed demonstrated acceptable precision, accuracy, linearity, detection sensitivity and stability.

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