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Chemical profiling of pharmaceuticals by capillary electrophoresis in the determination of drug origin

Cheryl L. Flurer*, Karen A. Wolnik

National Forensic Chemistry Center, US Food and Drug Administration, 1141 Central Parkway, Cincinnati, OH 45202, USA

Abstract

Capillary electrophoresis has been utilized to detect trace components in bulk pharmaceutical products, with emphasis on the identification of differences among manufacturers that can be used for source verification in suspect/counterfeit cases. Micellar electrokinetic capillary chromatography with sodium dodecyl sulfate was used in the analyses of β-lactam antibiotics. The aminoglycoside clindamycin phosphate and the macrolide erythromycin stearate were analyzed using borate buffers with direct UV detection. Methyl-β-cyclodextrin was used as a buffer additive in the erythromycin studies. Determination of product potency using peak area ratios has been demonstrated for ampicillin and clindamycin phosphate.

1. Introduction

The use of capillary electrophoresis (CE) and micellar electrokinetic capillary chromatography (MECC) to detect trace components, drug-related impurities, and degradation products within a single pharmaceutical product has been demonstrated for a variety of compounds [1–11]. During the course of sample analysis at the National Forensic Chemistry Center, we may be called upon to determine whether a finished product contains a drug supplied by one manufacturer or another, particularly in the area of counterfeit pharmaceuticals. The Food and Drug Administration has legal and scientific processes, such as the New Drug Application (NDA) and Abbreviated New Drug Application (ANDA), by which it approves new and generic drugs. However, the agency must ensure that drugs are produced only by approved manufacturers, and that the manufacturer adheres to the processes, formulations, and source of raw materials that have been approved.

The development of protocols that identify characteristic components in a bulk drug is essential in this task. In cases concerning β-lactam antibiotics, these procedures may utilize simple modifications of previously published MECC methods [4,12]. In other cases the pharamaceuticals of interest have not been studied by CE or MECC, except as the means by which compounds such as macrolide antibiotics have been introduced into a mass spectrometer [13].

The work presented in this paper summarizes efforts to detect differences in bulk products in order to distinguish among manufacturers. Fig. 1 shows the structures of the three β-lactam antibiotics, the aminoglycoside clindamycin phosphate, and the macrolide erythromycin stearate

* Corresponding author.

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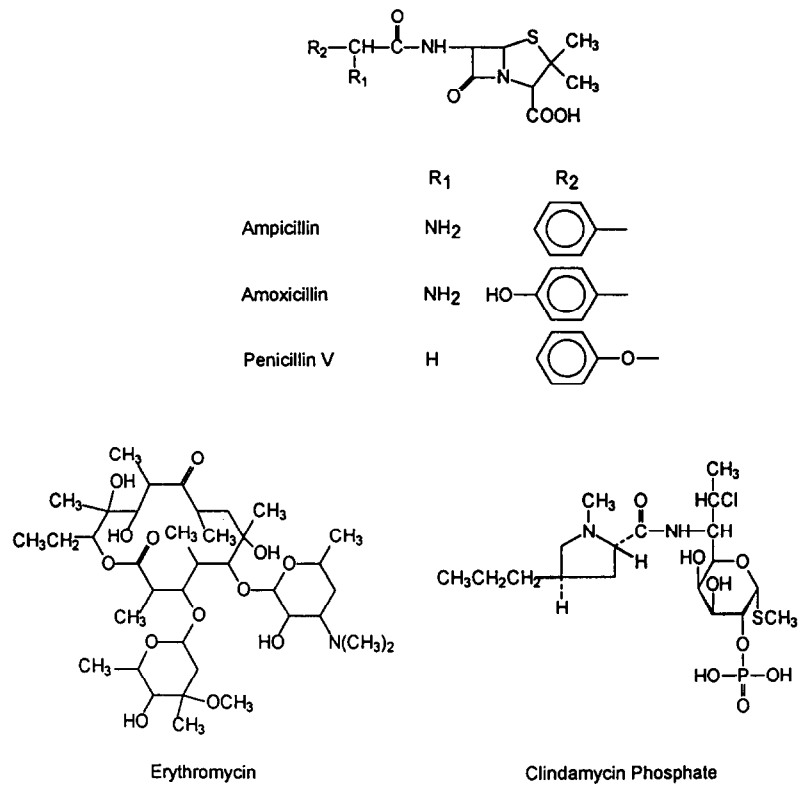

Fig. 1. Structures of antibiotics used in these studies.

that were the focus of studies being conducted in this laboratory. In the analyses of clindamycin phosphate and erythromycin stearate, the development of new separation protocols was required to enhance the minor differences observed among sample manufacturers.

2. Experimental

2.1. Reagents

Amoxicillin trihydrate (AMOX; lot H-1), penicillin V potassium (PENV; lot F-2), clindamycin phosphate (CLIPHOS; lot H), clindamycin hydrochloride (CLIND; lot G-1) and erythromycin stearate (ERYTHST; lot G-1) standards were purchased from US Pharmacopeial Convention (Rockville, MD, USA). Lincomycin hydrochloride (LINC; lot 51H05735), penicillin G potassium (PENG; lot 12H0275), penicillin V potassium (lot 20H0290), ampicillin anhydrous (lot 71H0594), and 6-aminopenicillanic acid (AMINOPEN; lot 30H3498) standards were purchased from Sigma (St. Louis, MO, USA). HPLC-grade isopropanol and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methyl-β-cyclodextrin (Me-β-CD) was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade. Distilled, deionized water was obtained in the laboratory from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Buffers were filtered through 0.2-μm nylon 66 filters (Alltech, Deerfield, IL, USA), and were degassed under aspirator vacuum. Other bulk drug samples used in these studies were available in the laboratory.

Fused-silica capillaries of 50 μm I.D. $\times$ 360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA), and Isco (Lincoln, NE, USA).

2.2. Instrumentation

For the study of the β-lactam antibiotics, a Dionex capillary electophoresis system with an Advanced Computer Interface (Dionex, Sunnyvale, CA, USA) was used, and data collection and processing were accomplished with the AI-450 chromatography software package. CLIPHOS and ERYTHST studies utilized an Isco Model 3140 Electropherograph. Data collection and processing were accomplished with the Isco capillary electrophoresis software package.

2.3. Sample analyses

β-Lactam antibiotics

Stock solutions of standards and samples were prepared in 1% phosphate buffer, pH 6, to concentrations of 2.0 mg/ml, and were diluted with the phosphate buffer to appropriate levels as necessary. A 0.20 mg/ml mixture of AMOX, ampicillin, PENG, AMINOPEN and PENV was used to standardize the system in terms of migration times and peak areas. Due to sample deterioration, all solutions were used within 3 h of preparation.

Samples were introduced into the capillary by gravity injection at a height of 100 mm for 15 s. Separations took place in an 80 cm capillary (75 cm to detector), using a 0.050 *M* sodium dodecyl sulfate (SDS)–0.10 *M* NaH_2PO_4–0.05 *M* $Na_2B_4O_7$, pH 8 buffer at +18 kV with 205 nm direct detection. Analyses were accomplished at ambient temperatures. The capillary was rinsed for 3 min between analyses with buffer.

For the profiling experiments, all samples were analyzed initially without spikes. The relative percentage composition of a component within a sample was calculated after normalizing the component peak areas with respect to their migration times [1]. Ampicillin samples of 50 μg/ml were quantitated with 20 μg/ml AMOX as an internal standard. Potency is given in terms of micrograms of anhydrous ampicillin per milligram of solid, so the appropriate adjustment in concentration was calculated if the sample was in the trihydrate form. A ratio of normalized peak areas of ampicillin/AMOX was used as a single point comparison between the Sigma ampicillin standard (920 μg/mg potency) and the sample.

Clindamycin phosphate

Sucrose was used as an internal standard in these studies. Samples of CLIPHOS and sucrose were dissolved in distilled water such that the sucrose concentration was 15 mg/ml and CLIPHOS was 0.18 mg/ml. Samples were introduced into the capillary by vacuum injection at 25.0 kPa s.

Separation was accomplished at 34°C with a 75 m*M* $Na_2B_4O_7$ buffer, pH 9, at +15 kV in a 70 cm capillary (45 cm to detector). Direct detection took place at 195 nm. The capillary was rinsed with buffer for 3 min between analyses. For the profiling experiments, all samples were analyzed initially without spikes. The potency of the CLIPHOS samples was determined with single point comparisons of the ratio of normalized peak areas of CLIPHOS/sucrose to the US Pharmacopeia (USP) CLIPHOS standard (799 μg clindamycin/mg clindamycin phosphate).

Erythromycin stearate

Samples were dissolved in isopropanol, and were diluted with distilled water to produce a 4.0 mg/mL ERYTHST solution in isopropanol–water (50:50). Due to sample deterioration, solutions were prepared daily. The buffer used was 60 m*M* $Na_2B_4O_7$–10 m*M* Me-β-CD–5% methanol, pH 9, and was prepared daily. Samples were introduced into the capillary by vacuum injection at 25.0 kPa s. Separation was accomplished over a 90 cm capillary (65 cm to detector) at +22 kV, with 205 nm direct detection at 34°C. The capillary was rinsed with buffer for 4 min between analyses.

3. Results and discussion

3.1. β-Lactam antibiotics

This family of antibiotics is perhaps the most widely used of all antibiotics available. Members of this family are also easily detected at UV wavelengths, and have been studied extensively

by CE and MECC as separate standards [4,12,14,15], in tablets and injectable solutions [7,15], in gastric contents [16] and in human plasma [17]. The method used in the present studies was a modification of the MECC buffers previously used [4,12]. Table 1 demonstrates the reproducibility of the separation of the standard mixture over six consecutive injections. The relative standard deviations (R.S.D.s) given for the migration times of the five standards are indicative of those observed with the components discussed below.

Fig. 2 shows a qualitative comparison of AMOX samples from three different manufacturers, and Table 2 summarizes the relative percentage compositions of the trace components of interest. The peak identified in Fig. 2 as a degradation product differs in initial quantity from one sample to another, and is essentially absent from manufacturer C (Fig. 2D). However, the intensity of this peak increases with solution age, and therefore cannot be used reliably as a point of differentiation. The presence of component 2 is an interesting feature of manufacturer C; the small peak present in the sample from manufacturer A at a similar migration time was not present consistently and was not evaluated. Additionally, the baseline distortion seen at 21 min represents a unique aspect of the amoxicillin samples obtained from manufacturer C.

Although samples from all manufacturers test-

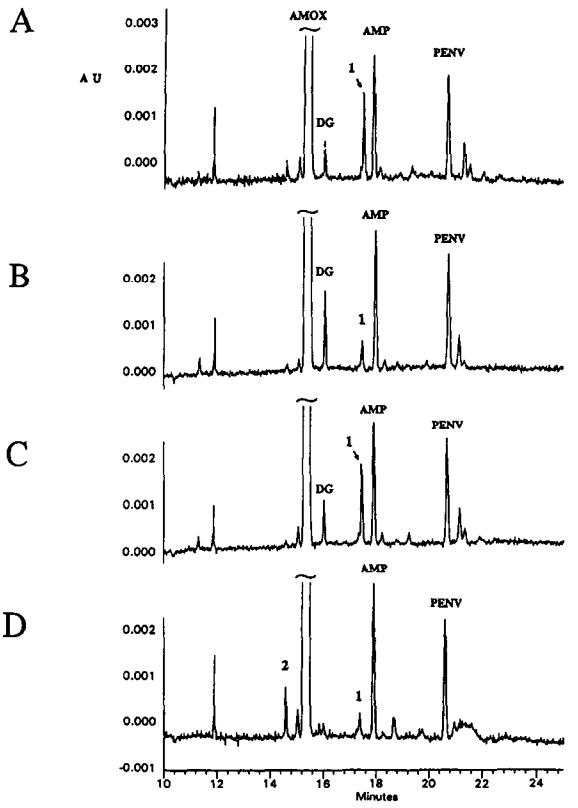

Fig. 2. Capillary electrophoretic analysis of 2.0 mg/ml AMOX samples: (A) manufacturer A; (B) manufacturer B, sample 1; (C) manufacturer B, sample 2; (D) manufacturer C. Separation conditions are given in text. DG = Degradation product; AMP = 20 μg/ml ampicillin spike; PENV = 20 μg/ml penicillin V potassium spike. Percentage composition of components 1 and 2 are given in Table 2.

Table 1
Reproducibility of β-lactam antibiotic standard mixture ($n = 6$)

	AMOX	Ampicillin	PENG	AMINOPEN	PENV
Migration time (min)					
Average	15.63	17.97	18.52	20.02	20.91
S.D.	± 0.12	± 0.12	± 0.12	± 0.14	± 0.14
R.S.D. (%)	0.78	0.67	0.65	0.68	0.68
Peak area					
Average	12 259	13 913	12 609	6552	12 363
S.D.	± 242	± 229	± 197	± 98	± 160
R.S.D. (%)	1.97	1.65	1.56	1.50	1.29

Table 2
Comparison of relative percentage composition of components in amoxicillin by manufacturer

	A[a]	B	C[b]
Component 1			
Average	0.544	0.0947[c]	0.160
S.D.	±0.050	± 0.0198	± 0.018
R.S.D. (%)	9.2	20.9	11
Average		0.904[d]	
S.D.		± 0.072	
R.S.D. (%)		8.0	
Component 2			
Average			0.342
S.D.			±0.030
R.S.D. (%)			8.8

Component numbers correspond to those given in Fig. 2.
[a] $n = 12$; three lots, four repetitions each.
[b] $n = 17$; repetitions from same lot.
[c] $n = 7$; two lots, four repetitions each; value of 0.042 discarded.
[d] $n = 12$; three lots, four repetitions each.

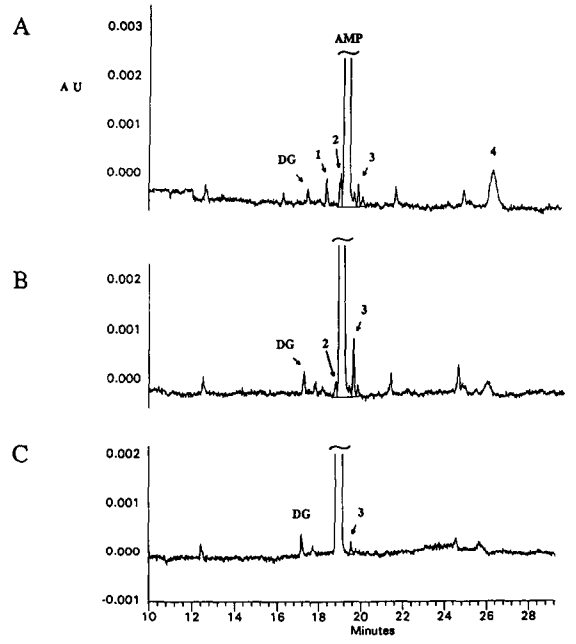

Fig. 3. Capillary electrophoretic analysis of 2.0 mg/ml ampicillin (AMP) samples: (A) manufacturer E; (B) manufacturer D; (C) manufacturer F. Separation conditions are given in text. DG = Degradation product. Percentage composition of components 1–4 are given in Table 3.

ed contain components corresponding to component 1 (Fig. 2), the differences in relative abundances between manufacturers are outside the standard deviations determined during analyses. They are sufficiently significant that they can be used as markers to distinguish between the lots analyzed from manufacturer A and the two sets of lots analyzed from manufacturer B (see Table 2).

Similar qualitative and quantitative comparisons can be made among ampicillin samples (Fig. 3 and Table 3) and PENV samples (Fig. 4 and Table 4) to facilitate the identification of a particular manufacturer. As was the case with manufacturer B, samples of ampicillin that were obtained from manufacturer E show some variation among lots. However, by utilizing the relative abundances of both components 2 and 3, the two sets of lots from manufacturer E can be distinguished from manufacturer D and from one another. In the case of the penicillin V samples, the relative cleanliness of the sample (Fig. 4C) as compared to the standards (Fig. 4A and B) can be used as identification.

The R.S.D.s reported in Tables 2–4 are not as small as those reported for components having relative abundances of ≤1% [1] or ≤0.1% [4] in other pharmaceutical preparations. Solutions of all three β-lactam antibiotics demonstrated nonlinear behavior at concentrations above 1.2 mg/ml, so the use of 2.0 mg/ml solutions could contribute to the poor R.S.D.s. However, the qualitative differences among samples were not as pronounced in solutions of 1.0 mg/ml, because the smaller peaks could not be distinguished from baseline fluctuations with confidence. For the purposes of this series of experiments, the relatively poor R.S.D.s were accepted, because differences between samples were outside the range of standard deviations established.

Quantitation of ampicillin using amoxicillin as an internal standard is given in Table 5. The USP method for potency determination is an HPLC procedure [18]. Both the USP and CE methods are straightforward and involve the use of peak area ratios. The potency values calcu-

Table 3
Comparison of relative percentage composition of components in ampicillin by manufacturer

	E	D	F
Component 1			
Average	0.313		
S.D.	± 0.026		
R.S.D. (%)	8.3		
Component 2			
Average	0.441	0.248	
S.D.	± 0.018	± 0.027	
R.S.D. (%)	4.1	11	
Average	0.192[a]		
S.D.	± 0.030		
R.S.D. (%)	16		
Component 3			
Average	0.191	0.564	0.077
S.D.	± 0.022	± 0.027	± 0.026
R.S.D. (%)	12	4.8	34
Average	0.368[a]		
S.D.	± 0.017		
R.S.D. (%)	4.6		
Component 4			
Average	1.143		
S.D.	± 0.106		
R.S.D. (%)	9.27		

Component numbers correspond to those given in Fig. 3; $n = 3$; repetitions from one sample lot, unless otherwise noted.
[a] $n = 6$; two lots, three repetitions each.

lated with the CE normalized peak area ratios are within 7% of the declared potency, as given by the supplier of the antibiotic using the USP procedure. Reproducibility of this method is given in Table 6, with assays performed over four days with fresh preparations of standards and unknowns, and two buffer preparations. It is interesting that the potency values obtained for the trihydrate preparations were consistently higher than their declared values, and that those for the anhydrous preparation were consistently lower. It is not known whether this behavior is indicative of a general trend, or whether it simply occurred with the three samples used in this study.

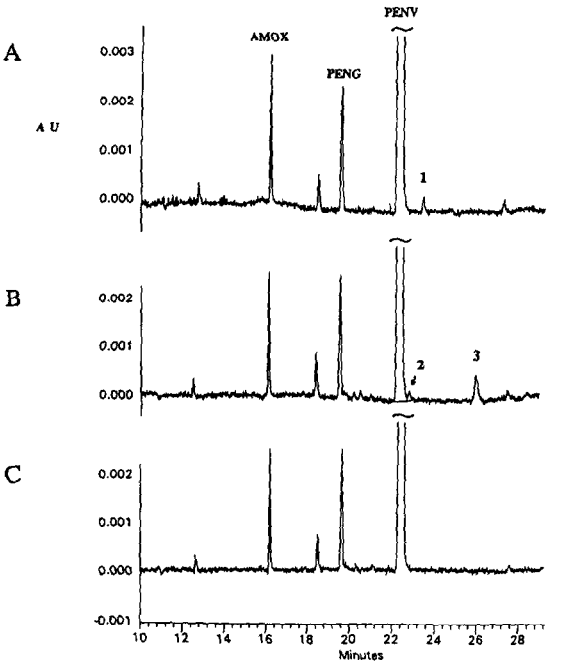

Fig. 4. Capillary electrophoretic analysis of 2.0 mg/ml PENV samples: (A) USP Standard; (B) Sigma standard; (C) manufacturer G. Separation conditions are given in text. AMOX = 20 μg/ml amoxicillin spike; PENG = 20 μg/ml penicillin G potassium spike. Percentage composition of components 1–3 are given in Table 4.

Table 4
Comparison of relative percentage composition of components in penicillin V potassium by supplier

	USP	Sigma
Component 1		
Average	0.126	
S.D.	±0.010	
R.S.D. (%)	7.9	
Component 2		
Average		0.095
S.D.		± 0.026
R.S.D. (%)		27
Component 3		
Average		0.424
S.D.		± 0.014
R.S.D. (%)		3.3

Component numbers correspond to those given in Fig. 4. $n = 4$.

Table 5
Quantitation of ampicillin

	Normalized peak areas ampicillin/AMOX	Quantitation (potency)[a]	
		Declared[b]	Calculated
Sigma Standard[c]			
Average	2.538	920	
S.D.	±0.066		
R.S.D. (%)	2.6		
Manufacturer D[d]			
Average	2.096	849	891
S.D.	±0.054		
R.S.D. (%)	2.6		
Manufacturer E[d]			
Average	2.164	867	914
S.D.	±0.071		
R.S.D. (%)	3.3		
Manufacturer F[c]			
Average	2.594	996	931
S.D.	±0.042		
R.S.D. (%)	1.6		

[a] Potency given in terms of micrograms of ampicillin per milligram of solid.
[b] Based on USP protocol [18] utilizing HPLC procedure.
[d] $n = 4$; preparation is anhydrous form.
[d] $n = 6$; preparation is trihydrate form.

3.2. Clindamycin phosphate

Clindamycin phosphate is the water-soluble ester of clindamycin, which, in turn, is a semi-synthetic derivative of lincomycin, produced by *Streptomyces lincolnensis* [19]. Clindamycin phosphate is used in the treatment of serious infections that are caused by susceptible anaerobic bacteria and strains of streptococci, pneumococci and staphylococci [20], and is reserved for penicillin-allergic patients. As stated by Ackermans *et al.* [21], CE studies of aminoglycoside

Table 6
Reproducibility of ampicillin potency determination

	Manufacturer		
	D	E	F
	Average potency values (μg/mg)[a]		
	891, 903, 884, 961[b]	914, 935, 921, 903	931, 970, 964, 947
Average	893	918	953
S.D.	± 10	± 13	± 18
R.S.D. (%)	1.1	1.4	1.9

[a] n between 4 and 6 per day.
[b] Value excluded.

antibiotics as a group have been avoided, because these antibiotics in general contain no chromophores, so direct detection is difficult. The use of imidazole for indirect detection and a cationic surfactant at low pH for analysis in an anodic mode has met with considerable success with regard to detection sensitivity and separation selectivity [21]. Unfortunately, for the work that we are called to do, this approach may not accentuate differences among manufacturers of the same antibiotic.

Previous work with mono-, di- and oligosaccharides [22,23] has demonstrated that borate buffers form UV-detectable complexes with hydroxyl moieties. As seen in Fig. 1, CLIPHOS contains a *cis*-1,2-diol configuration on the pyranose ring and should form a particularly stable complex [22]. The negatively charged complex should also alleviate interactions with the capillary wall, and permit analyses in the cathodic mode that were not previously successful [21].

Fig. 5 (trace A) demonstrates the detection of the USP standard CLIPHOS and the internal standard sucrose (peak 1), with efficiencies for CLIPHOS on the order of 200 000 plates/m. Similar behavior is seen with the sample from manufacturer H (Fig. 5, trace B). The migration times of sucrose (9.307 ± 0.020 min, $n = 14$) and

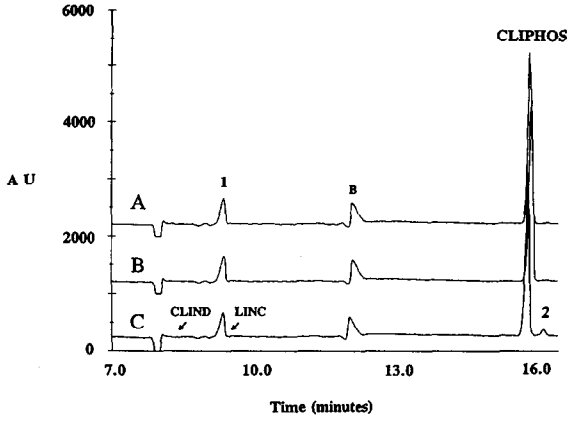

Fig. 5. Separation of internal standard sucrose (15 mg/ml; peak 1), CLIPHOS (0.18 mg/ml), and unknown (peak 2) from (A) USP Standard, (B) manufacturer H and (C) manufacturer J. Separation conditions are given in text. B = Buffer blank.

CLIPHOS (15.730 ± 0.019 min, $n = 14$) yield R.S.D.s of approximately 0.2%. Analysis of a sample from manufacturer J reveals the presence of an unidentified component (peak 2, Fig. 5, trace C) with a migration time of 16.053 ± 0.006 min ($n = 4$). Two reasonably expected impurities, clindamycin and lincomycin, migrate as indicated by the arrows in Fig. 5 (trace C). Although LINC and sucrose co-migrate under these buffer conditions, separate injections of the CLIPHOS samples without added sucrose do not indicate the presence of lincomycin. Two samples from different lots from manufacturer J yielded relative percentage compositions of the minor peak as 4.614 ± 0.121% and 4.024 ± 0.069%. There are indications of the presence of this unknown in the other samples, but it is not readily quantitated.

Table 7 summarizes the quantitation of CLIPHOS based on the use of sucrose as an internal standard. The USP method for potency determination of clindamycin phosphate is an HPLC procedure [24]. For the bulk drug, current USP guidelines require not less than 758 μg of clindamycin per mg of clindamycin phosphate, calculated on the anhydrous basis. As seen in Table 7, this CE methodology is 5% from the manufacturers' declared potency. The acceptable range for injectable solutions is 90–120% of the potency given on the product label, and 90–110% for topical solutions [24]. As was the case with ampicillin, the CE method given here is well within USP guidelines for product acceptance.

3.3. Erythromycin stearate

Erythromycin stearate is the stearic acid salt of the macrolide antibiotic erythromycin, which is used against many gram-positive bacteria [20]. Fig. 6 illustrates the presence of minor components within the USP standard and samples from two manufacturers. The migration times decreased over the course of the day, presumably due to the changing concentration of methanol at the elevated temperatures used in these experiments. However, the buffer is sufficiently stable to verify the presence of ERYTHST and

Table 7
Quantitation of clindamycin phosphate in terms of micrograms of clindamycin per milligram of clindamycin phosphate

	Peak area[a]		Area ratio CLIPHOS/Sucrose	Quantitation[b]	
	Sucrose	CLIPHOS		Declared	Area ratio
USP Standard					
Average	12 120	42 678	3.5255	799	
S.D.	± 240	± 310	±0.0732		
R.S.D. (%)	1.98	0.726	2.08		
Manufacturer H					
Average	12 382	46 164	3.7323	845	856
S.D.	± 299	± 513	±0.0785		
R.S.D. (%)	2.41	1.11	2.10		
Manufacturer J, sample 1					
Average	11 492	39 806	3.4661	828	789
S.D.	± 326	± 395	±0.0964		
R.S.D. (%)	2.84	0.992	2.78		
Manufacturer J, sample 2					
Average	11 667	41 626	3.5690	846	817
S.D.	± 228	± 275	±0.0738		
R.S.D. (%)	1.95	0.661	2.07		

$n = 8$ for all samples.
[a] Peak areas normalized during data acquisition.
[b] Calculated as ratio of USP response to sample.

to perform identification experiments. Component 1 is used primarily as a qualitative indicator, because the peak shape is too poor to give reproducible relative abundances, as indicated in Table 8. However, the absence/presence of component 1 definitely differentiates between the supplier of the USP product and the other manufacturers. Component 2 demonstrates good

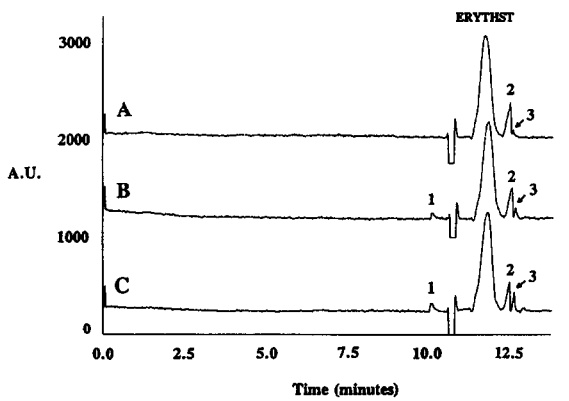

Fig. 6. Capillary electrophoretic analysis of 4.0 mg/ml ERYTHST samples: (A) USP Standard; (B) manufacturer K; (C) manufacturer L. Separation conditions are given in text. Percentage composition of components 1–3 are given in Table 8.

Table 8
Comparison of relative percentage composition of components in erythromycin stearate by manufacturer

	USP[a]	K[b]	L[b]
Component 1			
Average		3.570	1.936
S.D.		±0.190	± 0.293
R.S.D. (%)		5.3	15
Component 2			
Average	11.51	7.471	10.83
S.D.	± 0.32	±0.377	± 0.37
R.S.D. (%)	2.8	5.0	3.4
Component 3			
Average	1.27	3.450	2.233
S.D.	± 0.10	±0.296	± 0.173
R.S.D. (%)	7.9	8.6	7.8

[a] $n = 5$ from one lot.
[b] $n = 4$ from one lot.

reproducibility and can be used to distinguish among the USP supplier and manufacturers K and L, particularly when coupled with the relative abundance of component 3. Two additional lots from manufacturer L were studied. The relative abundances for component 2 were 11.27 and 11.15%, and for component 3, 4.148 and 3.038%. Using component 2, samples from manufacturers K and L can still be distinguished, despite the fact that component 3 is more variable and cannot be used in this instance.

4. Conclusions

The studies presented in this paper have demonstrated the ability to utilize the efficiency and flexibility inherent to CE and MECC to facilitate the differentiation among manufacturers of bulk pharmaceutical products. Reproducibility of these methods allows both qualitative and quantitative comparisons in terms of the relative percentage abundances of minor components. It is also possible to utilize CE methods to determine product potency, circumventing the need for multiple identification and quantitation assays called for in USP guidelines. As a matter of course, it will be necessary to determine whether the qualitative and quantitative aspects of this work will be applicable to finished products such as tablets, injectables, and ointments, which contain excipients that may interfere with some analyses.

The comparisons presented here are by no means exhaustive of all approved manufacturers of each of these pharmaceuticals. It is recognized that further refinements of the methods and data manipulations used may be required as the data base increases. With regard to differences that appear within samples from one manufacturer, we will need to establish a possible pattern between trace component profiles and dates of production, amended preparation procedures, etc. This pattern recognition would be facilitated by the identification of the impurities themselves. Additionally, verification will be necessary by comparing the presumed manufacturer's sample to an authentic sample, particularly to account for variations due to buffer preparation, capillary age, ambient temperature, and differences among lots from the same manufacturer. Although much work has yet to be done, the work presented here demonstrates that capillary electrophoretic methods present a viable approach to the cataloging of differences among manufacturers of bulk pharmaceuticals.

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