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Assessment of capillary electrophoresis in pharmaceutical applications

Analysis and quantification of a recombinant cytokine in an injectable dosage form

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ABSTRACT

Several methods are available for determining the components present in a typical pharmaceutical dosage form. Although some of these methods are simple and have received wide pharmaceutical industry acceptance over many years, several limitations still need to be overcome. Capillary electrophoresis was examined as an alternative method for the determination of a recombinant cytokine in a pharmaceutical dosage form. The results show that capillary electrophoresis can separate the recombinant cytokine from its dosage form matrix adequately, rapidly and with minimum sample volume requirements. Owing to its high efficiency, high resolution and simplicity, capillary electrophoresis appears to be very applicable to pharmaceutical samples. For reliable quantification of the resulting data, however, it is essential to optimize the experimental conditions, such as separation buffer and capillary column temperature. The advantages and potential of capillary electrophoresis for the separation of active ingredients and excipients in pharmaceutical formulations are discussed.

INTRODUCTION

Many techniques have been developed for the assay and quality control of proteins and peptides in pharmaceutical formulations, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), bioassay, polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) (for a review, see refs. 1 and 2). Each of these techniques, however, has certain limitations, including the complexity and time-consuming nature of assay development, the relatively large amounts of sample required to reach a reasonable level of sensitivity and the high degree of assay variability. Consequently, we have evaluated capillary electrophoresis (CE), which is capable of high resolution and high detection sensitivity, as a complementary technique for the analysis of pharmaceutical formulations. In CE, a high-voltage electric field induced across a small-bore fused-silica capillary provides

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the driving force needed to separate the components of the assay sample [3–6]. The inherently high resolving power of CE not only permits the separation of the protein or peptide from its formulation excipients, but also makes it possible to assess minute changes in protein structure (*e.g.*, deamidation, oxidation) which might have profound effects on drug stability and bioactivity.

In this work, we examined the use of CE to separate and quantify two recombinant human cytokines (leukocyte A interferon and interleukin-1 α) in a formulation mixture and in an actual pharmaceutical dosage form.

EXPERIMENTAL

Reagents and samples

High-purity potassium hydroxide, sodium phosphate, sodium tetraborate, hydrochloric acid and lithium chloride were obtained from Sigma (St. Louis, MO, USA). Millex disposable filter units (0.22 μm) were purchased from Millipore (Bedford, MA, USA). Fused-silica capillary columns were obtained from Scientific Glass Engineering (Austin, TX, USA) and Polymicro Technologies (Phoenix, AZ, USA). Recombinant human leukocyte A interferon and recombinant interleukin-1 α purified bulk drug substances were prepared at Hoffmann-La Roche (Nutley, NJ, USA). Human serum albumin (Albuminar-25) was purchased from Armour Pharmaceutical (Kankake, IL, USA) and N-acetyltryptophan was obtained from Cyclo Chemical, a division of Travenol Labs. (Los Angeles, CA, USA). Triply distilled, deionized water was used for the preparation of buffer solutions. Capillary electrophoresis buffers were routinely degassed and sonicated under vacuum after filtration.

Assay samples

Formulation mixtures of recombinant leukocyte A interferon (400 μg/ml), recombinant interleukin-1 α (200 μg/ml) and a combination of recombinant leukocyte A interferon (400 μg/ml) and recombinant interleukin-1 α (200 μg/ml) were prepared by adding the appropriate amounts of the purified bulk drug substance to 0.05 *M* sodium phosphate buffer solution (pH 7.0) containing 500 μg/ml of human serum albumin (HSA) and 50 μg/ml of N-acetyltryptophan. A typical pharmaceutical liquid dosage form of recombinant human interleukin-1 α (200 μg/ml) was also prepared and used for CE analysis.

Instrumentation

The initial separation experiments were performed using a laboratory-made capillary electrophoresis apparatus similar to that described previously [3,4]. Samples were injected electrokinetically and the electropherograms were recorded and integrated using a Model L-6512 strip-chart recorder (Linseis, Princeton Junction, NJ, USA) at 20 cm/h, and a Model 3390 integrator (Hewlett-Packard, Avondale, PA, USA). For the controlled-temperature experiment a commercial instrument was used (P/ACE System 2000; Beckman Instruments, Palo Alto, CA, USA). In this instrument, the capillary is housed in a cartridge configured to allow a flow of recirculating liquid with Peltier temperature control of the capillary. Samples were injected by pressure and the data acquisition and analysis were carried out using System Gold Chromatography Software (Beckman Instruments, San Ramon, CA, USA).

Procedure

The CE separations were performed using a 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* lithium chloride. Alternative buffers used previously [3,4], such as 0.05 *M* sodium phosphate buffer (pH 7.0) and 0.05 *M* sodium tetraborate buffer (pH 8.3), were also used. Samples to be assayed were transferred to the appropriate application vessel, *i.e.*, a 1.5-ml microcentrifuge tube or P/ACE sample holder, and applied to the capillary as described below. On completion of each run, the capillary column was cleaned by injection of 0.1 *M* sodium hydroxide solution, followed by distilled water, and then regenerated with fresh buffer solution.

Running conditions

For the laboratory-made instrument, a 89 cm (41 cm to the detector) × 75 μm I.D. capillary column was used. Samples were injected electrokinetically at 10 kV for 12 s and then separated at 13 kV at ambient temperature. Under these conditions, *ca.* 12 nl of solution were injected into the capillary column. Monitoring of the samples was carried out at 210 nm.

For the P/ACE system 2000, a 57 cm (50 cm to the detector) × 75 μm I.D. capillary column was used. Samples were injected by a positive nitrogen pressure of 3500 Pa for 3 s and then separated at 10 kV at 20, 25 or 30°C. Under these conditions, *ca.* 15 nl of solution were injected into the capillary column. Monitoring of the samples was carried out at 200 nm.

RESULTS

Fig. 1 depicts the electropherograms of the two cytokine formulations tested: recombinant human leukocyte A interferon in a formulation mixture containing HSA and N-acetyltryptophan, and recombinant human interleukin-1 α in a pharmaceutical dosage form containing the same excipients. Both the leukocyte A interferon (peak 1) and the interleukin-1 α (peak 2) were well separated from the excipients HSA (peak 3) and N-acetyltryptophan (peak 4). Further, the separation and resolution demonstrated in these electropherograms were maintained when the formulation containing the interleukin-1 α was spiked with a comparable amount of leukocyte A interferon prior to analysis (Fig. 2).

The observed electropherograms for both cytokine samples were complex, indicating the presence of major and minor component peaks migrating in addition to the main components identified. It is interesting that leukocyte A interferon, which routinely assays at greater than 95% purity by PAGE and HPLC, was resolved into two peaks by CE. At present, the nature of the separation achieved and the identity of the second peak component are unknown. For the interleukin-1 α sample, the additional peak eluting just after the main cytokine peak most likely corresponds to its deamidation byproduct.

Buffer composition was observed to play an important role in the selectivity (*i.e.*, resolution and peak sharpeness) of separation. Although sodium phosphate buffer (pH 7.0) and sodium tetraborate buffer (pH 8.3) were able to resolve the various analytes present in the two kinds of samples (data not shown), an increased selectivity was apparent with the sodium tetraborate buffer when it was supplemented with 0.025 *M* lithium chloride. We are currently investigating the effects of salts such as lithium chloride in the separation of proteins and peptides by CE.

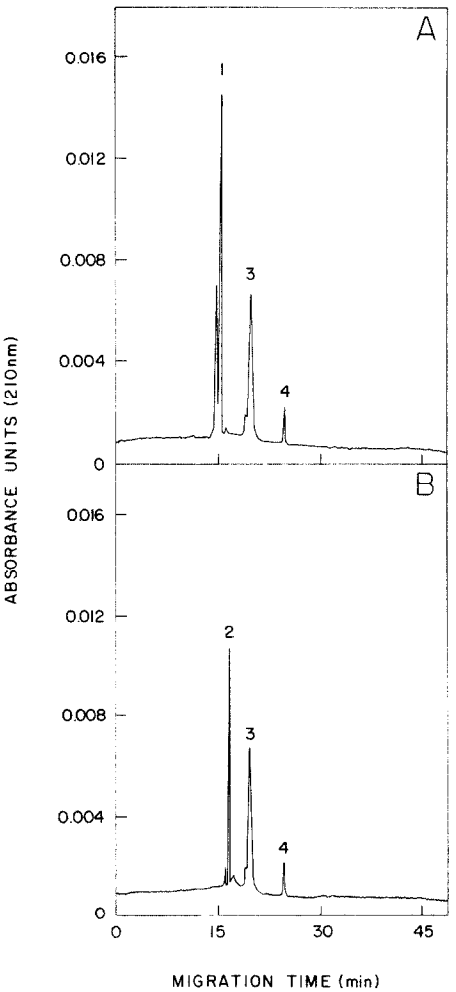

Fig. 1. Capillary electrophoresis profile of the cytokines interferon and interleukin. (A) Electropherogram of a formulation mixture containing recombinant leukocyte A interferon (peak 1), human serum albumin (peak 3) and N-acetyltryptophan (peak 4). (B) Electropherogram of an actual pharmaceutical dosage form containing recombinant interleukin-1 α (peak 2), human serum albumin (peak 3) and N-acetyltryptophan (peak 4).

In order to test the reproducibility when using the simple laboratory-made instrument at ambient running temperature, a solution of HSA was assayed six times over a period of *ca.* 4 h. As in the standard assay procedure, cleaning of the capillary column was carried out after every injection. Albumin was selected as a model protein as it is known to have an affinity for glass and might conceivably bind to the walls of the capillary column. Proteins in general are known to adsorb to fused silica [7]. As shown in Table I, the reproducibility of the electropherograms for both migration time and peak area recovery was excellent.

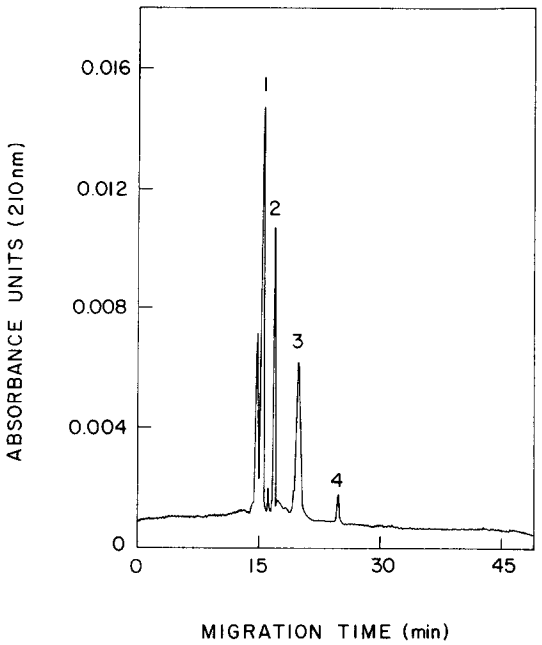

Fig. 2. Capillary electrophoresis profile of an actual pharmaceutical dosage form containing recombinant interleukin-1 α (peak 2), human serum albumin (peak 3), N-acetyltryptophan (peak 4) and spiked with recombinant leukocyte A interferon (peak 1).

The effect of variations in running temperature on the CE analysis was also investigated. Thermal experiments were performed using the P/ACE System 2000 CE instrument, which is constructed so as to allow fine temperature control of the capillary column. Separations were performed at 20, 25 and 30°C. The results of the temperature study are shown in Fig. 3 and Table II. The data shows that when the

TABLE I

PRECISION OF MIGRATION TIME AND ELECTROPHEROGRAM PEAK AREA FOR HUMAN SERUM ALBUMIN

Run No.	Migration time (s)	Peak area (units)
1	1489	15.77
2	1478	15.02
3	1487	16.52
4	1473	15.98
5	1483	16.09
6	1476	15.25
Mean	1481.00	15.77
Standard deviation	6.36	0.56
Relative standard deviation (%)	0.43	3.52

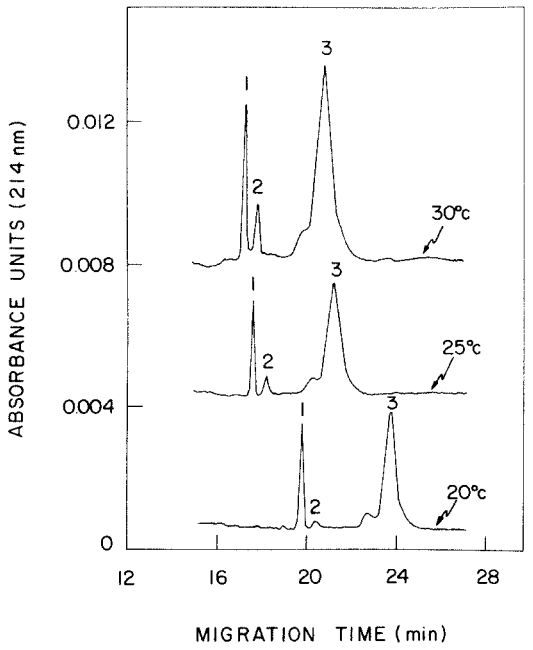

Fig. 3. Effect of temperature on migration times and peak areas of the sample components when subjected to 20, 25 and 30°C during separation. Electropherogram of an actual pharmaceutical dosage form containing recombinant interleukin-1 α, human serum albumin and N-acetyltryptophan. Peak 1 represents the intact interleukin-1 α, peak 2 its putative deamidation byproduct and peak 3 human serum albumin.

TABLE II

EFFECT OF TEMPERATURE ON THE MIGRATION TIMES AND PEAK AREAS OF ANALYTES BY CAPILLARY ELECTROPHORESIS

Temperature (°C)	Substance	Migration time (s)	Peak area (normalized percentage)
20	Interleukin	1178	20.32
	Deamidation product	1214	1.77
	Albumin	1425	77.91
25	Interleukin	1045	15.32
	Deamidation product	1081	3.27
	Albumin	1262	81.41
30	Interleukin	1020	12.59
	Deamidation product	1056	4.20
	Albumin	1232	83.21

temperature of the capillary column was increased the proteins migrated more rapidly, resulting in shorter separation times. The effects of an elevated running temperature on resolution and analyte recovery, however, were more complex. This is evident in the case of HSA (Fig. 3, peak 3), which at higher temperature shows an increase in peak area but a decrease in resolution. The interleukin-1 α (Fig. 3, peak 1), on the other hand, shows a decrease in recovery at higher temperature accompanied by an increase in the proportion of peak 2, its putative deamidation byproduct. On the basis of these findings, *i.e.*, diminished resolution and decreased stability, the use of an enhanced running temperature for these samples appears to be undesirable.

The effects of running conditions on the CE analysis are reflected in the observed relative standard deviations, which ranged between 0.4% and 2.0% for migration times and between 1.9% and 5.5% for peak areas, depending on the nature of the buffer, the separation temperature and the kind of instrument used (data not shown). The importance of temperature control in capillary electrophoresis for maintaining high resolution of the analytes and high efficiency of the technique has been described previously [8,9].

As interleukin-1 α apparently undergoes a temperature-dependent degradation in the capillary column during CE analysis, an attempt was made to generate a calibration graph for this cytokine under the most adverse conditions (30°C). Despite the enhanced degradation of the protein in the capillary column, the detection signal (peak area units) appears to be linear up to a cytokine concentration of 100 μg/ml. At the higher concentration tested (200 μg/ml), the observed peak area was slightly lower than expected. Hence, quantification of interleukin-1 α by CE appears to be at least feasible.

DISCUSSION

Protein and peptide components of a pharmaceutical formulation must be analyzed with an appropriate biological and/or chemical assay. The chemical assay of a protein or peptide drug substance in a pharmaceutical dosage form presents several unique problems. In general, pharmaceutical and diagnostic products are not present simply as pure substances but rather as complex formulated mixtures. Often, several chemical components or excipients are added for specific purposes, such as to enhance stability during storage, or to provide protection through a manufacturing process step (*i.e.*, freeze-drying or sterile filtration). Many of the excipients used in the formulations, however, are themselves proteinaceous in nature. In addition, because of the inherently strong biological potency of most of the protein and peptide drugs, the dosage forms usually contain only a few micrograms of the active drug. Commonly these drug substances must be protected by milligram amounts of excipients.

For a chemical assay, therefore, the method used should have sufficient sensitivity for measuring the low levels of drug substance in the dosage form and, at the same time, have the resolving power necessary to separate it from large amounts of excipients. Further, the method must have the consistent linearity required to encompass all dosage strengths expected. Unfortunately, many of the procedures currently available for the analysis of proteins and peptides in pharmaceutical dosage forms have some limitations. The assay methods used are frequently characterized as being nonspecific for the protein of interest, imprecise, subject to interference by other sub-

stances present, limited in sensitivity or non-linear over the range desired. Capillary electrophoresis, a method that offers many unique features, such as high resolution, high mass sensitivity (minimum sample requirements) and simplicity, may be able to address some of these limitations.

The experiments described here demonstrate that CE has the sensitivity and degree of resolution required for the analysis of two representative cytokines, interleukin-1 α and leukocyte A interferon, in typical formulation mixtures. Both cytokines were well separated from their excipients in a relatively short time and with a minimum sample requirement. The resulting electropherograms generated with either the simple CE apparatus, operating under ambient temperature conditions, or with the more sophisticated CE unit, using Peltier temperature control of the capillary column and operating at a specific isothermal temperature, show excellent reproducibility.

Capillary column temperature was observed to have an important influence on the electrophoretic results. With interleukin-1 α, for example, an enhanced operating temperature contributed to a faster migration rate resulting in a shorter separation time of the cytokine. Higher temperatures, however, also resulted in an increased degree of degradation of the cytokine, presumably through a deamidation pathway. Temperature influences diverse factors such as electric current, viscosity of the electrophoretic media, ionization of the migrating species, degree of electroosmotic flow, and the extent of surface adsorption of components to the capillary column. Each of these factors may contribute, to varying extents, to the overall characteristics of the run. Elevation of the running temperature in CE with increased current has been associated with parallel decreases in the viscosity of the electrophoretic buffer and in the efficiency of separation [8]. A higher running temperature may also affect the stability of the protein being analyzed. Deamidation of asparagine and/or glutamine residues is one of the most common non-enzymatic covalent modifications of proteins [10–15], and it is known that the rate of deamidation increases with temperature, and in the presence of certain buffer ions [11].

Finally, the CE running temperature seems to influence the recovery of proteins from the capillary column, as represented by relative absorbance (peak area). It is possible that at lower separation temperatures, protein adheres to the walls of the capillary more readily than at higher separation temperatures. On the other hand, it may also be possible that the amount of sample injected into the capillary column varies according to the method of injection and the temperature of the system. As a consequence, system and method differences may have a marked effect on the quantification of data. For samples such as interleukin-1 α dosage form, strict control of the temperature of the capillary column during separation is essential in order to apply CE as a quantitative analytical method. This is critical as the separation profile, and stability of the protein, appear to be influenced by as little as a 5°C variation in temperature.

CONCLUSIONS

Capillary electrophoresis has been clearly demonstrated to be a promising method for the separation of active ingredients and excipients in pharmaceutical formulations. Further, the incorporation of a temperature control system (during the

separation of analytes) is an important factor for maintaining constant column resistance, optimizing separation efficiency, reducing sample decomposition and maintaining a desired chemical equilibrium. The control of temperature is an essential parameter to yield an acceptable degree of reproducibility of the migration times during the analysis of the active ingredients of a dosage form. For quantification of proteins such as interleukin-1 α, which might be subject to degradation during separation, the running conditions must be modified to minimize degradation and appropriate standards developed. When the additional work has been completed, capillary electrophoresis should complement, if not surpass, existing methodology for the investigation of proteins in pharmaceutical dosage forms.

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