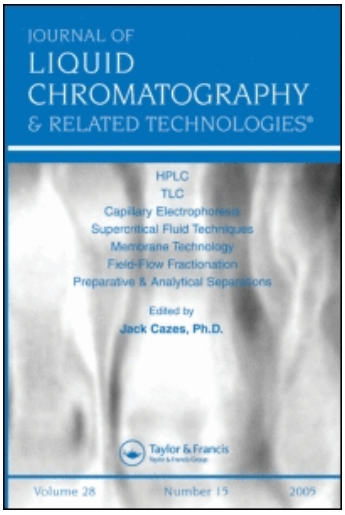

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THE EFFECT OF OPERATING PARAMETERS ON THE ANALYSIS OF A HUMAN ALPHA-INTERFERON BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

The influence of the operating parameters in capillary zone electrophoresis (CZE) on the separation of a human alpha-interferon has been investigated. The separation was characterised by the electrophoretic mobility of the first and last observed peaks, and the number of peaks on the electropherogram, whether visible as shoulders or adequately resolved. No peaks could be observed at lower pH values (below pH 4.7) due to capillary wall adsorption. The optimum pH (pH 6.1) for the CZE analysis of human alpha-interferon was close to the isoelectric points of the sample components (pI 5.5 - 6.5). The investigation of the effect of the borate, phosphate and Trizma buffer concentration on the separation showed that increasing buffer concentration generally decreases the electrophoretic mobility with increased resolution, up to a certain optimum. Longer capillary lengths and slightly higher voltages also increased the resolution. The maximum number of peaks (17) was observed by using 200 mM phosphate buffer (pH 6.1) with 50 cm effective length capillary applying 12 kV voltage.

INTRODUCTION

WELLFERON* (interferon alfa-n1 [lns]) is a highly purified blend of natural human alpha interferons obtained from human (Namalwa) lymphoblastoid cells following induction with Sendai virus [1] and purified by a multistep process [2]. It is used in the treatment of hairy cell leukemia and chronic hepatitis B and non-A, non-B (c) infection. Clinical studies have yielded encouraging results in the treatment of papillomatosis. Preliminary studies are in progress to investigate its effect on delaying the onset or the progress of the acquired immune deficiency syndrome (AIDS), particularly when used in combination with RETROVIR* (zidovudine).

The protein content of alpha interferon has been extensively studied by Zoon et. al. [3-5], and the components have been separated by sequential immunoabsorbent affinity chromatography followed by ultrafiltration and reversed phase high performance liquid chromatography (RP-HPLC). WELLFERON can contain at least twenty-two subtypes, each of which contains 164 -166 amino acids. The apparent molecular weights ranged from 17,500 to 23,000 Da on non-reducing SDS-PAGE and 17,500 to 27,600 Da using reducing SDS-PAGE. The components were sequenced by Edman degradation and compared to those found previously from cDNA or genomic DNA sequences [6, 7]. There was also evidence for glycosylation of one of the subtypes, which contained glucosamine, galactosamine, fucose and/or mannose and galactose [8].

The RP-HPLC analysis of WELLFERON [3] also showed the complexity of the sample, as 11 peaks (many of them only as shoulders) appeared on the chromatogram obtained from a Vydac C-4 column with an acetonitrile gradient and 0.1% TFA in the mobile phase.

High performance capillary zone electrophoresis (CZE) provides a fast, gentle, and non-denaturing separation conditions for the analysis of high molecular weight proteins. It can therefore offer

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an alternative to RP-HPLC for the further analysis of the human alpha-interferon. The resolution in CZE can be increased by changing the pH [9], and the effect of the applied voltage, buffer type and concentration on the resolution has been investigated in several studies [10-12]. A guide for optimisation of resolution in CZE has been given by Atamna et. al. [13].

In this study, the effect of buffer type and concentration, pH, column length and the applied voltage on the separation of the human alpha-interferon components by CZE have been investigated.

EXPERIMENTAL

The WELLFERON sample was obtained "in house". The samples were analysed at concentrations of 0.1 - 0.5 mg/ml diluted with deionised water from a 3 mg/ml sample.

An ISCO Model 3850 capillary electropherograph was used for the CZE analysis (ISCO Inc., Lincoln, Nebraska, U. S. A.). Samples (8µl) were manually injected using a 10 µl syringe, and split 1:1930, so that 4 nl samples were injected onto the column. The separation was monitored using a UV detector at 200 nm. Uncoated fused silica capillaries, with 50 µm internal diameters were obtained from ISCO Inc., and were cut to lengths of 61 cm and 80 cm giving 36 cm and 50 cm effective lengths, respectively. The capillaries were rinsed with deionised water, 0.1M NaOH and deionised water after each buffer change and rinsed with buffer prior to each analysis. The capillaries were stored in 0.1M NaOH overnight.

Analytical grade disodium tetraborate, sodium dihydrogen orthophosphate, disodium hydrogen phosphate, sodium acetate trihydrate, acetic and phosphoric acid were obtained from BDH (Poole, Dorset, U. K.), the Trizma-base and Trizma-HCl from Sigma (Poole, Dorset, U. K.). The deionised water used was obtained from a Millipore Milli Q Plus water purification system. The concentration and pH of the various buffers are summarised in Table 1.

TABLE 1.

The Buffers Used for the CZE Analysis of WELLFERON.

Buffer	Concentration (mM)	pH
1. Borate	20-170	9.02-9.98
2. Phosphate	50-250	6.01-7.29
3. Phosphate	150	3.05
4. Acetate	50-100	4.64-4.72
5. Acetate-phosphate	100-250	4.18-4.37
6. Trizma	50-300	7.40-8.48

The buffers were prepared from the appropriate salt by adjusting the pH with the appropriate acid or base. They were filtered using a 0.4 μm Millipore Millex-HA filter before use.

The conditions optimised were applied voltage, buffer type, concentration and pH. Initially a 36 cm effective length capillary was used with the longer 50 cm effective length being used when the optimum buffer conditions had been found.

The separation was characterised by the mobility of the first and last components which takes into account the variation in applied voltage. The electrophoretic mobility was calculated according to the following formula:

$$\mu = \frac{l.L}{t.V}$$

where μ is the mobility ($cm^2sec^{-1}V^{-1}$), l is the effective capillary length (cm), L is the total length (cm), t is the migration time (sec), V is the applied voltage. The number of peaks, whether visible as shoulders or adequately resolved on the electropherogram were also used for characterising the resolution and the selectivity obtained by varying operating parameters, since the peaks could not been identified and followed from one condition to the other.

RESULTS AND DISCUSSION

Using buffers 3 - 5 (see Table 1) at pH's lower than 4.72, no peaks could be detected on the electropherogram. At the low pH the sample is positively charged, (the isoelectric point of WELLFERON components are in the range of 5.5 to 6.5 [14]) and can adsorb irreversibly onto the negatively charged capillary wall [15]. Therefore, buffers with higher pH than the isoelectric point were used for optimising the separation.

The effect of buffer concentration with three different buffers and pH on the electrophoretic mobility of the first and last peak, together with the number of peaks separated are summarised in Tables 2 - 4.

It can be seen from the data that increasing buffer concentration in general decreased the electrophoretic mobility of the WELLFERON components, which is in agreement with the general theory [10-12]. The resolution also increased as the higher ionic strengths minimise analyte interactions with the silica capillary wall and maintain constant conductivity and pH within the analyte zone, so preventing band broadening. However, high conductivities and currents can produce more heat thus causing band broadening and decreasing resolution [15-16], which results in the decrease in the number of peaks at high buffer concentrations, thus lower mobility does not necessarily result in higher resolution.

The optimum borate buffer concentration was 150 mM pH 9.3 which separated 14 components over an 11 - 15 min period. The electropherogram obtained under these conditions is shown in Fig. 1.

The optimum phosphate buffer concentration was 200 mM with pH 6.1 by which 15 components were separated in 21 - 35 min. The electropherogram can be seen in Fig. 2.

The optimum Trizma buffer concentration was 250 mM, pH 7.9 by which 15 components were separated in 18 - 29 min. The electropherogram obtained under these conditions is shown in Fig. 3. The Trizma buffer contains zwitterions which do not contribute significantly to the overall conductivity, this allows the application of high voltages without the problem of generating excessive heat.

TABLE 2.

The Electrophoretic Mobility of the First and Last Peaks and the Number of Peaks on the Electropherogram of WELLFERON at pH 9.3±0.2 with respect to the Borate Buffer Concentration. Effective Capillary Length 36 cm.

Concentration (mM)	μ, mobility of the first peak	last peak	Number of Peaks
20	4.7	-	1
50	4.1	3.3	6
100	3.4	2.8	8
120	2.8	2.3	9
150	2.2	1.7	14
170	2.0	1.6	14

TABLE 3.

The Electrophoretic Mobility of the First and Last Peaks and the Number of Peaks on the Electropherogram of WELLFERON at pH 6.5±0.2 with respect to the Phosphate Buffer Concentration. Effective Capillary Length 36 cm.

Concentration (mM)	μ, mobility of the first peak	last peak	Number of Peaks
50	4.7	3.6	7
100	3.2	2.1	7
150	2.6	1.8	14
175	2.4	1.6	14
200	2.3	1.7	15
225	2.0	1.6	13
250	2.2	1.8	11

TABLE 4.

The Electrophoretic Mobility of the First and Last Peaks and the Number of Peaks on the Electropherogram of WELLFERON at pH 8.0±0.1 with respect to the Trizma Buffer Concentration. Effective Capillary Length 36 cm.

Concentration (mM)	μ, mobility of the first peak	last peak	Number of Peaks
50	3.8	3.5	4
100	3.1	2.7	6
200	2.3	1.7	12
250	2.0	1.4	15
300	1.9	1.4	14

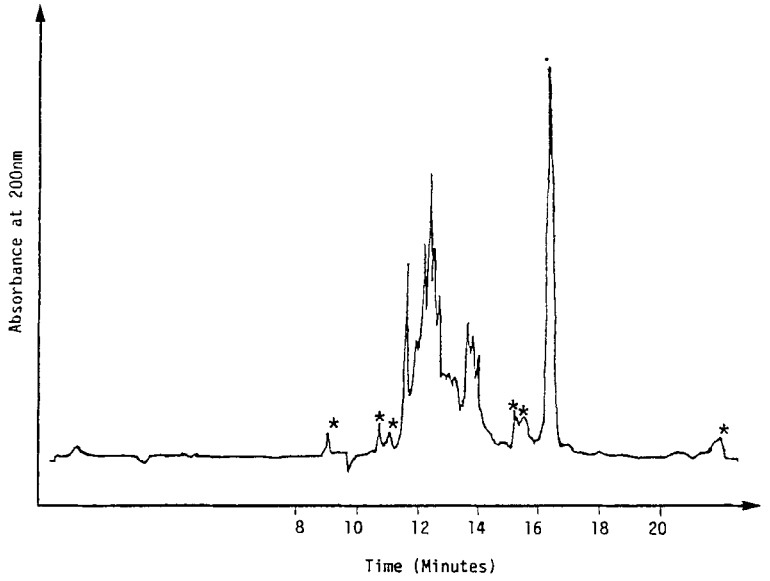

FIGURE 1. Electropherogram of human alpha-interferon. Effective capillary length 36 cm with 150 mM borate buffer pH 9.3. Applied voltage was 15 kV and the current was 93 μA. The peaks marked with * were found also in the blank sample.

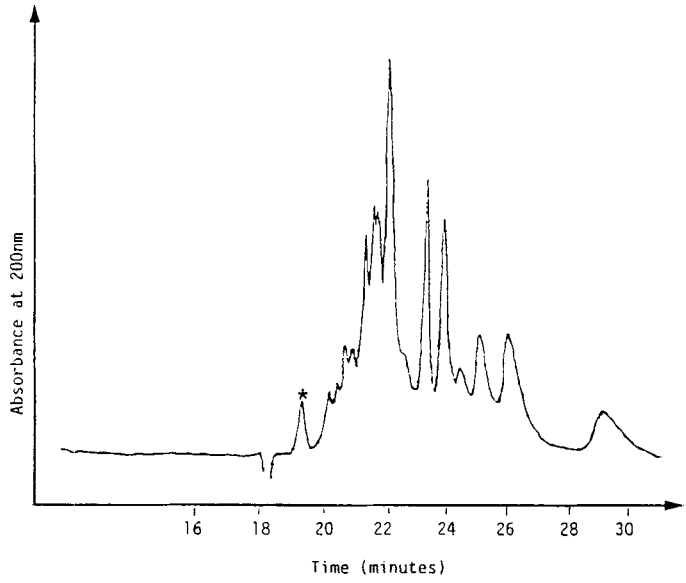

FIGURE 2. Electropherogram of human alpha-interferon. Effective capillary length 36 cm with 200 mM phosphate buffer pH 6.1. Applied voltage was 8 kV and the current was 42 μA. The peak marked with * was found also in the blank sample.

The overall effect of the buffer concentration, at a given pH, on the number of peaks observed has been summarised in Figure 4.

Table 5 shows the effect of pH and buffer type on the mobility of the first and last peaks and the number of peaks on the electropherogram using 100 mM buffer concentrations.

The mobility of the first peak did not depend significantly on the pH and buffer type. The mobility of the last peak was lowest when phosphate buffer with pH 6.5 was used. This pH is the closest to the isoelectric point of the proteins in the WELLFERON sample. In the range of the isoelectric points some of the components can be in positively charged form, while others are not, thereby differentiating between the sample components with similar shapes and sizes. A pH

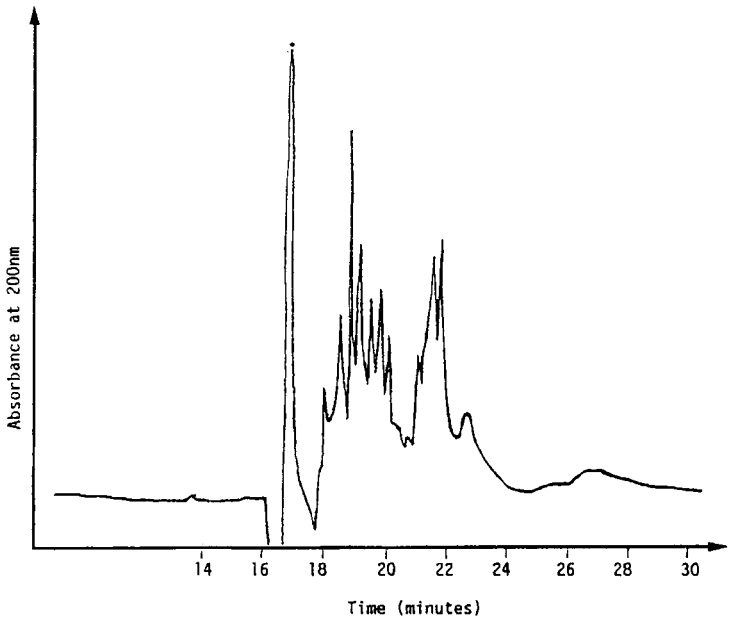

FIGURE 3. Electropherogram of human alpha-interferon. Effective capillary length 36 cm with 250 mM Trizma buffer pH 7.9. Applied voltage was 10 kV and the current was 50 μA. The peak marked with * was found also in the blank sample.

TABLE 5.

The Electrophoretic Mobility of the First and Last Peaks and the Number of Peaks on the Electropherogram of WELLFERON by Using 100 mM Borate, Phosphate and Trizma Buffers.

Buffer	pH	μ, mobility of the first peak	last peak	Number of peaks
Borate	9.3±0.2	3.4	2.8	8
Phosphagte	6.5±0.2	3.2	2.1	7
Trizma	8.0±0.1	3.1	2.7	6

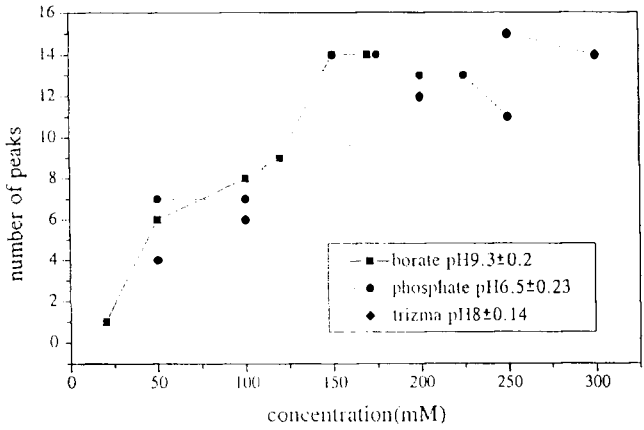

FIGURE 4. The plot of the number of peaks on the electropherogram of human alpha-interferon as a function of buffer concentration.

TABLE 6.

The effect of the capillary length and the applied voltage on the separation of the WELLFERON components with various buffers.

Buffer	Effective Capillary Length					
	36cm			50cm		
	No. of Peaks	Voltage (kV)	Current (μA)	No. of Peaks	Voltage (kV)	Current (μA)
150mM Borate pH 9.31	14	15	93	15	15	67
150mM Borate pH 9.5	12	16	100	13	15	68
200mM phosphate pH 6.12	15	9	55	17	12	42
200mM phosphate pH 6.01	15	8	42	16	15	49
250mM Trizma pH 7.9	15	10	50	14	14	50
300mM Trizma pH 7.96	14	8	46	15	15	65

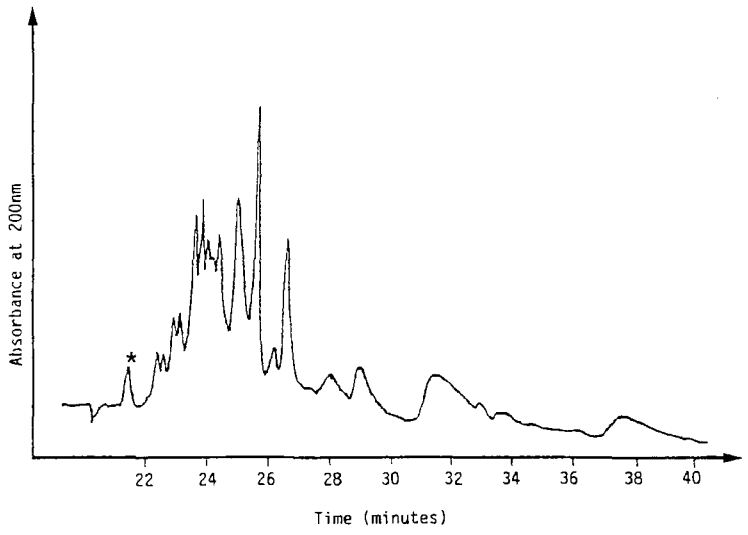

FIGURE 5. Electropherogram of human alpha-interferon obtained by using 50 cm effective capillary length with 200 mM phosphate buffer pH 6.1. Applied voltage was 12 kV and the current was 42 μA. The peak marked with * was found also in the blank sample.

value near to the midpoint of the isoelectric point range will thus tend to maximise the components net charge differences [17], therefore providing the widest range in their mobilities.

For further optimisation of the separation of the multicomponent WELLFERON sample, the effect of the capillary length and the applied voltage have been investigated by using the three types of buffer at the optimum concentration. As found earlier [13], the resolution can be improved by increasing the voltage up to a certain point, avoiding the heating effect and thus the zone broadening. By increasing the capillary length the migration time and the column efficiency can be increased without changing the selectivity. The separation was characterised by the number of detectable peaks on the electropherogram. Table 6 shows the data

obtained. The greatest number of peaks (17) was obtained by using 200 mM phosphate buffer pH 6.12 with a 50 cm effective length capillary applying 12 kV voltage. The electropherogram obtained is shown in Figure 5.

In conclusion, the protein components of WELLFERON could be best separated at pH 6.1, which is within the isoelectric point range. The increasing buffer concentrations decreased the mobility of the components and increased the peak resolution up to a certain point. The longer effective capillary length with a slightly higher applied voltage also improved the separation of the sample components.

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