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Analysis of a vaccine purification process by capillary electrophoresis

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ABSTRACT

Free-zone capillary electrophoresis (CZE) was applied to the analysis of samples from the individual purification steps in the production of a licensed vaccine product. With this technique, real-time analysis can be performed to ensure that purification parameters are being met before proceeding to the next step. In addition to monitoring the product peak, a unique pattern of associated host cell contaminants also is displayed. This unique "fingerprint" at each step of the purification process may have practical utility for production in that it demonstrates lot-to-lot consistency in the manufacturing process of the vaccine.

INTRODUCTION

Recombinant products are produced in a host organism such as bakers' yeast (*Saccharomyces cerevisiae*). Whether the product is secreted into the growth medium or retained intracellularly, a reproducible purification process is required to bring the product to an acceptable level of purity. Typically, such a process is monitored at various stages by specific assays which may be based on serological reactivity, bioactivity or mass (*e.g.* UV or protein measurements). Although these assays can provide specific information about the functioning of the purification process, they give limited information about the reproducibility of the process. Therefore, other assays are required in order to measure impurities and the addition or removal of reagents used in the purification and formulation of the product.

We have evaluated the use of free-zone capillary electrophoresis (CZE) [1] for monitoring the purification of the recombinant hepatitis B vaccine expressed in *S. cerevisiae* (Recombivax HB®). Others have reported the use of CZE to measure the homogeneity and purity of final recombinant products [2,3]. Here we report the use of CZE to follow the purification of Recombivax HB by analyzing each step in the purification process for the amount of product, removal of impurities and additions and removal of reagents, thus establishing a 'fingerprint' for the entire process run.

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EXPERIMENTAL

Instrumentation

CZE analysis of the Recombivax HB production process was performed in an Applied Biosystems (Foster City, CA, USA) Model 270A capillary electrophoresis system using an uncoated open capillary 72 cm long (50 cm working length) × 50 μm I.D.

Materials

Samples for direct analysis from various stages in the production process for Recombivax HB and a thimerosal concentrate, used as a standard, were obtained from Merck Pharmaceutical (West Point, PA, USA). Triton X-100 (Triton), used as a standard, was purchased from Rohm and Haas (Philadelphia, PA, USA).

Capillary zone electrophoresis

All samples were analyzed at 30°C using 25 m*M* sodium phosphate running buffer at pH 7.25. A 27-kV electric field was applied across the capillary, with the detector end of the capillary being at a negative potential with respect to the inlet of the capillary. A typical running current was *ca.* 30 μA. Sample injection time was 1.5 s using vacuum, which results in a *ca.* 15-nl sample. A 2-min wash with 0.1 *M* sodium hydroxide solution followed by a 3-min wash with running buffer preceded each sample injection. Sample detection was by measurement of UV absorption at 200 nm.

RESULTS

Recombivax HB consists of 22-nm spherical lipoprotein particles of *ca.* $2 \cdot 10^6$ relative molecular mass (MW). Known as hepatitis B surface antigen (HBsAg) [4,5], each particle consists of *ca.* 100 226-amino acid polypeptides. Production of the polypeptides and assembly into HBsAg particles occurs within the recombinant yeast cells. The following is a partial list of the steps in the purification of HBsAg.

(A) Cell lysate. Yeast cells are ruptured and diluted in Triton.

(B) Filtered lysate. The *cell lysate* is clarified by microfiltration allowing the HBsAg to pass into the filtrate.

(C) Concentrated lysate. The *filtered lysate* is concentrated tenfold on a 100 kilodalton MW cut-off filter and Triton is removed by extraction with Amberlite.

(D) Silica product. The HBsAg is adsorbed on silica and eluted with borate buffer. It then is concentrated on a 100 kilodalton MW cut-off filter.

(E) HIC product. The HBsAg is adsorbed to a hydrophobic interaction chromatographic (HIC) column, then eluted with Triton in buffer.

(F) Amberlite product. The HBsAg-rich fractions from the HIC column are pooled and treated with Amberlite to remove Triton.

(G) Diafiltered Amberlite product. The *Amberlite product* is diafiltered with buffer over a 100 kilodalton MW cut-off filter.

(H) Thiocyanate product. The HBsAg product is diafiltered with 3 *M* potassium thiocyanate followed by buffer on a 100 kilodalton MW cut-off filter.

(I) Sterile Product. The pure HBsAg is diluted and sterile filtered.

(J) Formaldehyde product. The HBsAg product is treated with formalin and thimerosal is added as a preservative.

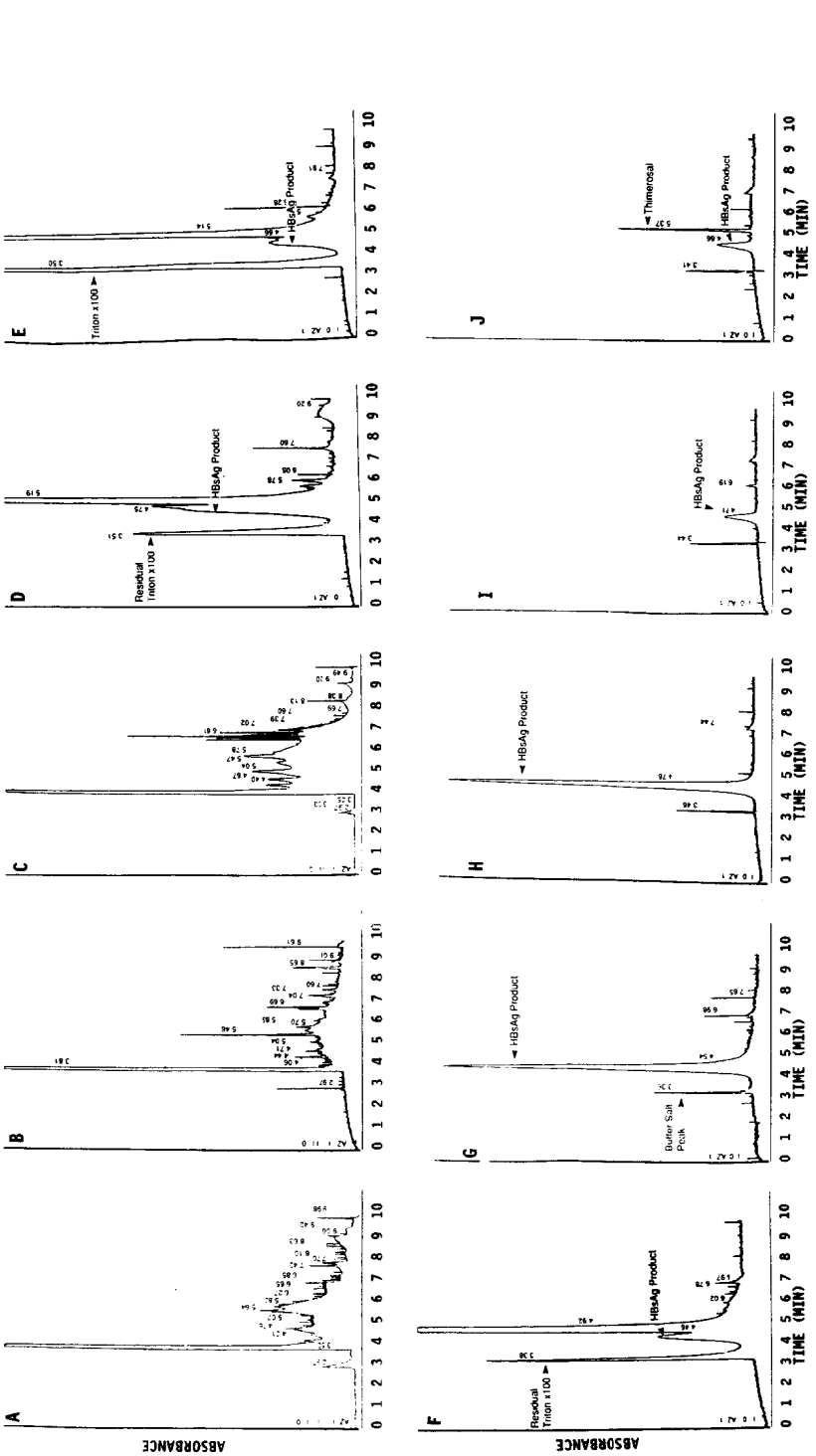
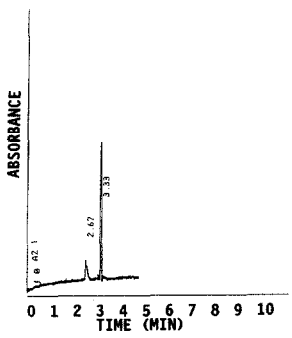


Fig. 1. Electropherograms of Recombivax HB hepatitis B vaccine production process steps. All electropherograms are plotted at attenuation 16 except for *cell lysate* and *concentrated lysate*, which are plotted at attenuation 128. (A) Cell lysate; (B) filtered lysate; (C) concentrated lysate; (D) silica product; (E) HIC product; (F) Amberlite product; (G) diafiltered Amberlite product; (H) thiocyanate product; (I) sterile product; (J) formaldehyde product.



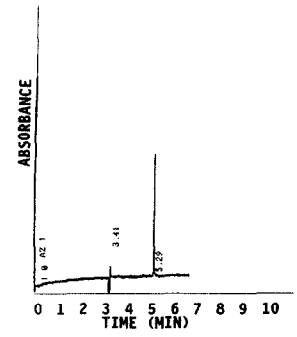


Fig. 2. Electropherogram of Triton X-100 at 50 μg/ml.

Fig. 3. Electropherogram of thimerosal at 16 μg/ml.

Fig. 1 shows ten individual electropherograms representing the above ten Recombivax HB production process steps. The *cell lysate* sample is crude, with both soluble and insoluble components. The *filtered lysate* shows that much of the protein has been removed by dilution and filtration. The *concentrated lysate* reflects a tenfold concentration. After the *silica product*, the protein load is greatly reduced. To facilitate direct comparison between electropherograms, all the remaining steps are plotted at the same attenuation. The first peak in the *silica product* electropherogram (3.51 min) is residual Triton. The following double peak (4.75 and 4.83 min) represents impurities and HBsAg. The large peak (5.19 min) is a buffer component with several small trailing impurity peaks. The *HIC product* electropherogram shows the addition of Triton (3.50 min). The double peak representing impurities and HBsAg is present as is the buffer component peak. The *Amberlite product* electropherogram shows reduction of the Triton peak (3.38 min). The HBsAg peak preceding the buffer component peak remains and the trailing impurity peaks are absent. The *diafiltered Amberlite product* electropherogram shows the removal of residual Triton as well as the buffer component compared with the previous electropherogram; additional impurities are also removed. The salt peak from the buffer used in the production process is resolved (3.36 min) and appears throughout the remainder of the process steps. The HBsAG product elutes 4.54 min. The *thiocyanate product* shows no change in the electropherogram from the preceding step. The *sterile product* electropherogram reflects a dilution made at this step with the HBsAg product at 4.71 min and the buffer salt peak at 3.44 min. The *formaldehyde product* electropherogram looks similar to the preceding electropherogram; however, it does show the addition of thimerosal (5.37 min).

Triton X-100, a commonly used detergent, can be determined by CZE at 3.3 min after injection. Fig. 2 shows a typical Triton peak at 3.33 min and represents an injection of 50 μg/ml of Triton. Linearity is good from 10 μg/ml and up to 10 mg/ml with a correlation coefficient (r) for a straight-line fit of 0.99900. The limit of detection is 10 μg/ml.

Thimerosal, a mercury-based preservative used in vaccine manufacture, can also be determined by CZE. Fig. 3 is an electropherogram showing thimerosal with a 16 μg/ml injection passing the detector at 5.29 min. The calibration graph shows good

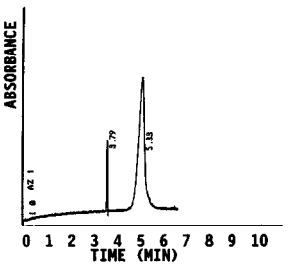


Fig. 4. Electropherogram of hepatitis B HBsAg at 100 μg/ml.

linearity from 3.2 μg/ml to 10 mg/ml with an r value for a straight-line fit of 0.99999. The limit of detection is 3μg/ml.

The recombinant HBsAg, which is the immunologically active component of Recombivax HB can be determined by CZE. Fig. 4 is an electropherogram showing a 100 μg/ml injection of HBsAg eluting at 5.33 min. A calibration graph for HBsAg shows excellent linearity between 5 and 200 μg/ml with an r of 0.99890.

DISCUSSION

There are several advantages of the use of CZE in the quality control of a production process, and they are evident as the Recombivax HB process is analyzed. A significant benefit which applies to the CZE analysis of all the process steps is that the results are available in less than 20 min. Quality control procedures currently used in the process consist of individual chemical and serological assays. The results of these assays typically follow one or more days after a particular process step has been completed. This time lag decreases process flexibility for action if a deviation is indicated. Additionally, each assay provides only a small "window" on the process step rather than the complete "fingerprint" that CZE analysis provides. With CZE analysis many types of problems can be identified and action taken while the process is ongoing. From an instrumentation point of view, CZE is very simple. A plain uncoated capillary is used, which can process hundreds of samples with virtually no maintenance. In our hands such an instrument can stand idle for days and be turned on, then samples processed within 20 min. The characteristics of this instrument are well suited to process monitoring.

(A) The *cell lysate* electropherogram is complex. It provides a "fingerprint" representing the initial yeast cell breakage. A significant change in the pattern would indicate an incomplete breakage of the yeast cells. With attenuation 128, Triton itself cannot be seen at the concentration used in the process. The large leading peak in the electropherogram is some component of the broken yeast cells. A large number of spiked peaks are apparent across the electropherogram and are attributed to insoluble particulates that flow by the detector.

(B) The *filtered lysate* step uses microfiltration, which allows the HBsAg product to pass through but retains large particulates. This electropherogram is analyzed at attenuation 16 as the bulk of the mass from the previous step has been removed by the filter. If the filter in this step were to have a partial or complete loss of integrity, it would be apparent from the "fingerprint", and corrective action could be taken.

Filter blockage also could be detected. The HBsAg cannot be seen at this step because it is too low in concentration.

(C) The electropherogram of the *concentrated lysate* reflects a tenfold concentration over a 100 000-dalton MW cutoff filter of the *filtered lysate* as well as passage across an Amberlite column to remove Triton added at the *cell lysate* step. Since the attenuation setting is high (128) to accommodate the concentration increase, residual Triton cannot be detected. The HBsAg cannot be identified definitively at this point in the process, but it is likely represented by the peak at 4.67 min.

(D) The *silica product* step retains the HBsAg, which then is eluted and analyzed. The first peak at 3.51 min is interpreted to be residual Triton from the *concentrated lysate* step, which bound to the Amberlite and subsequently coeluted with the HBsAg. The following double peaks are impurities and HBsAg product. The large peak (5.19 min) is a buffer component with some small trailing impurity peaks. The attenuation used to plot this electropherogram and all of the following electropherograms is 16.

(E) A hydrophobic interaction process step is used to further purify the HBsAg as *HIC product*. The HBsAg is removed using Triton. The first peak (3.50 min) reflects the addition of Triton and the second smaller peak (4.66 min) reflects the HBsAg product seen in the preceding electropherogram. The buffer component at 4.92 min remains. The trailing impurity peaks also have been reduced. Any problem with the affinity column or the addition of Triton would be detected easily by this analysis.

(F) The *Amberlite product* step is designed to remove most of the Triton from the product stream. The electropherogram for this step shows a substantial reduction in the Triton peak from its predecessor. The peaks for HBsAg and buffer component remain substantially unchanged. Any difficulties in this step would be apparent as a substantially larger Triton peak or perhaps a reduced HBsAg peak. Residual Triton and the remaining impurities are reduced by diafiltration over a 100 000-dalton MW cut-off filter.

(G) This *diafiltered Amberlite product* electropherogram shows the HBsAg peak (4.54 min) with the buffer component peak from the *Amberlite product* step removed. The remaining buffer salt peak (3.36 min) runs at the same location as Triton; after Triton removal, the salt peak becomes apparent.

(H) The *thiocyanate product* step consists of a 3-*M* potassium thiocyanate diafiltration followed by buffer diafiltration, which results in more complete disulfide bond formation in the HBsAg particle. The electropherogram remains unchanged from the preceding electropherogram, as might be expected.

(I) The *sterile product* step is the result of a dilution in buffer of the *thiocyanate product* material. As a result, the height and area of the HBsAg peak (20 μg/ml) are greatly reduced. The salt peak (3.44 min) remains as expected as the dilution was performed in buffer.

(J) The final step in this process, *formaldehyde product*, involves formalin treatment followed by the addition of the preservative thimerosal. This addition is represented by a peak at 5.37 min at this step. The HBsAg and the salt peaks are also present (4.66 and 3.41 min, respectively). From the electropherogram, the addition of thimerosal can be confirmed and determined, as can HBsAg, from calibration graph.

As demonstrated by the calibration graphs for Triton, thimerosal and HBsAg,

determination of a variety of macromolecules using CZE is possible. Questions about reproducible sample injection of very small volumes (*ca.* 15–30 nl) and flow-rates past the detector have been raised as possible problems. There are a significant number of laboratory-made CZE instruments in the field which may have contributed to the perception that quantification by CZE might be a problem. We did not find these issues apparent in our analyses.

While there is some variability in the migration time of HBsAg from step to step in the analysis process, this variability is small and without apparent trend. The variability seen may be due to instrument factors or to the association of HBsAg with various components of the process which might affect its migration time.

No significant shift in migration time is seen after potassium thiocyanate treatment. We understand the thiocyanate to be acting as a chaotropic agent, allowing for reformation of the disulfide bonds in the surface antigen particle with no change in charge. Additionally no significant change in migration time is seen after formaldehyde treatment, as one might expect owing to reactions with the free amino groups of arginine and lysine. There are seven such amino acids in each of the 100 polypeptides making up the HBsAg particle. Many of these may be within the interior of the HBsAg and unavailable for reaction. The size of HBsAg suggest that endosmotic flow may be the largest contributing factor to its migration.

In conclusion, CZE is a powerful and practical new tool for monitoring protein purification processes. It not only provides real-time analytical data to aid in control of the process but also provides the opportunity for intervention to correct problems that arise on a real-time basis. In addition, it provides a 'fingerprint' of each step, showing impurity removal and product enhancement and confirming additions of reagents where performed. Accumulation of composite process 'fingerprints' over a number of runs can demonstrate process reproducibility, which will have value for commercial products.

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REFERENCES

1 B. L. Karger, A. S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585–614.
2 R. G. Nielson and E. C. Rickard, *ACS Symp. Ser.*, 434 (1990) 36–49.
3 J. Frenz, S.-L.- Wu and W. S. Hancock, *J. Chromatogr.*, 480 (1989) 379–391.
4 D. Ganem and H. E. Varnus, *Ann. Rev. Biochem.*, 56 (1987) 651–693.
5 P. J. Kniskern, A. Hagopian, P. Burke, N. Dunn, D. L. Montgomery, L. D. Schultz, C. A. Schulman, C. E. Carty, R. Z. Maigetter, D. E. Wampler, E. D. Lehnman, S. Yamazaki, D. J. Kubek, E. A. Emini, W. J. Miller, W. M. Hurni and R. W. Ellis, in M. Z. Atassi (Editor), *The Application of Molecular Biology to the Development of Novel Vaccines. Immunobiology of Proteins and Peptides. 5. Vaccine Mechanism, Design, and Application (Advances in Experimental Medicine and Biology*, Vol. 251), Plenum Press, New York, London, 1989, pp. 83–98.