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Determination of free carrier protein in protein–polysaccharide conjugate vaccines by micellar electrokinetic chromatography

David H. Lamb[a,*], Luke Summa[a], Q. Paula Lei[a], Guillaume Duval[b], Olivier Adam[b]

[a]*Aventis Pasteur, Discovery Drive, Swiftwater, PA 18370-0187, USA*

[b]*Aventis Pasteur, Campus Mérieux, 1541 avenue Marcel Mérieux, 69 280 Marcy l'Etoile, France*

Abstract

Protein–polysaccharide conjugate vaccines offer the prospect of reducing morbidity and mortality due to bacterial pneumonia and meningitis but, because of their size and heterogeneity, are often a challenge to characterize by traditional analytical methods. Vaccines consisting of *Streptococcus pneumoniae,* or *Neisseria meningiditis* polysaccharide covalently linked to formaldehyde-inactivated diphtheria toxoid carrier protein were resolved from non-conjugated toxoid by micellar electrokinetic chromatography. Separation was achieved using alkaline sodium borate solutions containing sodium dodecyl sulfate in excess of the critical micellar concentration. No sample pretreatment was required prior to analysis. Diphtheria toxoid peak migration times were highly reproducible. Measurement of absolute toxoid peak area showed poor precision, but good precision was observed when peak area was normalized against an internal standard (myoglobin). Good linearity was observed over useful ranges of both protein content and injection time. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Purified polysaccharide vaccines provide protection in adults and older children against a number of bacterial diseases such as those caused by *Haemophilus influenzae, Streptococcus pneumoniae* and *Neisseria menigitidis* [1–3]. However, polysaccharide vaccines do not elicit T-cell dependent immune responses and are not effective in providing protection to infants, the population that suffers the effects of these illnesses most acutely [4]. The lack of a T-cell dependent response also limits the ability of most polysaccharides to induce immunological memory — a desirable outcome of vaccination. It is possible to overcome these deficiencies by covalently linking polysaccharides to carrier proteins to form T-cell dependent, protein–polysaccharide conjugate vaccines [5,6]. For example, vaccines in which poly(ribose-ribitol-phosphate) from *H. influenzae* (HIB) is conjugated to a suitable carrier have been licensed for use in infants in many countries and have dramatically reduced the incidence of HIB disease world wide. Considering the success of HIB vaccines, it is not surprising that vaccine manufacturers are actively pursuing development and licensure of conjugate vaccines against other bacterial pathogens.

Linking polysaccharides to protein carriers produces vaccines with heterogeneous populations of large (M_r 10^5–10^6) molecules. Sources of heterogeneity include variability in the length and number

*Corresponding author. Tel.: +1-570-839-4429; fax: +1-570-839-0619.

E-mail address: david.lamb@aventis.com (D.H. Lamb).

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of polysaccharide chains attached to individual carrier protein molecules and often, variability in the number of attachment sites for each polysaccharide chain [7]. Some conjugates, such as those described in this study, are produced using diphtheria (Dt) or tetanus (Tt) toxoids as carrier proteins. These toxoids are produced by inactivation of toxins with low levels of formaldehyde and may therefore contain intra- and inter-chain bridging [8] as well as modification of primary amines and various amino acid side chain groups [8,9]. Both Dt and Tt have been used as vaccines for decades and have an excellent history of safety and immunogenicity [10,11], properties that recommend their use as carrier proteins, but the intrinsic heterogeneity of toxoids and conjugate vaccines provides challenges for biochemical characterization by traditional methods.

Micellar electrokinetic chromatography (MEKC) [12] separates both by charge density and relative hydrophobicity. Hydrophobic separation is accomplished by the inclusion of a surfactant, such as cetyltrimethylammonium bromide (CTAB) or sodium dodecyl sulfate (SDS), at a level above the critical micellar concentration. MEKC is suitable for the analysis of biological material and has been used to study model proteins [13], peptides [14,15], human globulin chains [16], interferon-γ [17], and monoclonal antibodies [18–20]. MEKC of proteins has been discussed in several reviews [21–23].

The prior use of MEKC for glycoprotein analysis and the lack of analyte size restriction in capillary electrophoresis compared to acrylamide and agarose gel electrophoresis led us to explore the utility of MEKC as a method for examining protein–polysaccharide conjugate vaccines. To the best of our knowledge, no previously published studies have described MEKC analysis of either protein–polysaccharide conjugate vaccines or Dt or Tt. This report specifically examines the detection and quantitation of free diphtheria toxoid carrier protein in the presence of toxoid–polysaccharide conjugate vaccines. The method is simple to perform, requires no sample pretreatment and has been applied to toxoid conjugates prepared with polysaccharides from several serotypes of *S. pneumoniae* and *N. menigitidis*. This method may therefore be a useful tool for monitoring consistency of protein incorporation into protein–polysaccharide conjugates during routine vaccine manufacturing.

2. Experimental

2.1. Samples and reagents

Diphtheria toxoid (Dt) and tetanus toxoid (Tt) were produced by Aventis Pasteur. For experiments intended to quantitate Dt or examine assay reproducibility, Dt was dialyzed into 0.85% (w/v) sodium chloride to remove thimerosal preservative which forms a peak that co-migrates with Dt under many of the conditions tested (data not shown). The toxoid content after dialysis was estimated using the method of Bradford [24] and represents the mean of testing two separate toxoid dilutions in triplicate. Protein–polysaccharide conjugate vaccines were prepared by Aventis Pasteur Development Department and contained $\approx$1–3 mg protein and 0.5–1 mg polysaccharide per ml. Conjugate vaccines did not contain thimerosal preservative and were tested by capillary electrophoresis without prior treatment. Pneumococcal polysaccharide powders were prepared by the Aventis Pasteur Development Department and dissolved in 0.85% (w/v) saline to a final concentration of $\approx$2–2.5 mg/ml prior to MEKC analysis. SDS was practical grade from J.T. Baker (Phillipsburg, NJ, USA) or analytical grade from USB (Cleveland, OH, USA). Other chemicals used were reagent grade or better from J.T. Baker or Sigma (St. Louis, MO, USA).

2.2. Micellar electrokinetic chromatography

MEKC was performed using a Beckman-Coulter (Palo Alto, CA, USA) P/ACE model 5510 with a Beckman-Coulter UV detector. Uncoated fused-silica capillaries were from Polymicro (Phoenix, AZ, USA). The total capillary length was 67 cm (60 cm from inlet to detector). Separation buffer was prepared fresh the day of use by mixing 15 ml 0.05 *M* sodium borate, 2.5 ml 0.2 *M* SDS and 2.5 ml Milli-Q purified water. The final pH of the separation buffer was $\approx$9.15–9.25 and was not further adjusted. The capillary was rinsed prior to each separation with

Milli-Q purified water, 30% (v/v) methanol and 0.1 *M* NaOH (10 min each) followed by a 3-min rinse with separation buffer. Lyophilized myoglobin (Sigma) was reconstituted according to manufacturer's instructions. For use as an internal standard, 2 μl was added to 200 μl of sample.

2.3. Free solution capillary electrophoresis

Free solution capillary electrophoresis (FSCE) was performed using a Beckman-Coulter (France) P/ACE model 5500 with a Beckman-Coulter UV detector. Capillaries were as described above. Prior to each separation, the capillary was rinsed with water, 0.1 *M* NaOH, water and separation buffer (3 min each).

3. Results and discussion

3.1. Separation of free carrier protein

The goal of this study was to develop a method that would allow separation and quantitation of free carrier protein in protein–polysaccharide conjugate vaccines in order to assess manufacturing consistency of protein incorporation during the conjugation reaction. Alkaline (e.g. Tris–borate and glycine–NaOH) and neutral (phosphate) buffers at various concentrations, but without added surfactant, were tested for their usefulness in this separation. Successful separation of carrier protein from protein–polysaccharide conjugate material was observed for some, but not all, conjugates prepared using polysaccharides from pneumococcal and meningococcal

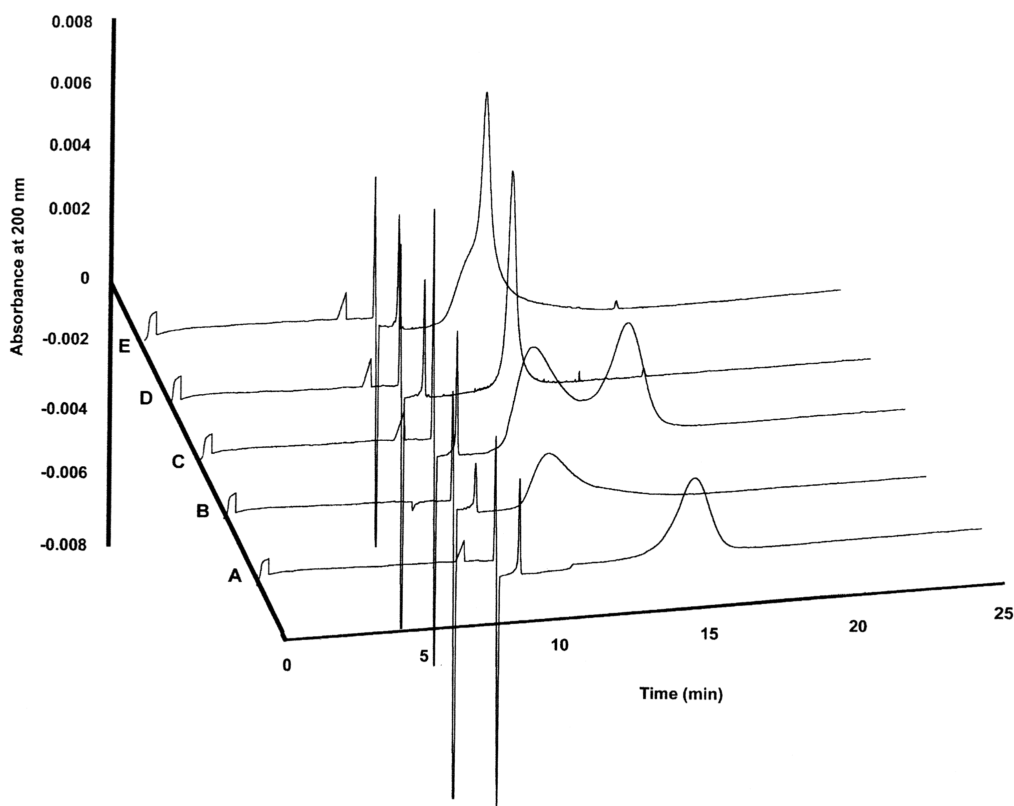

Fig. 1. Separation of Tt and two Tt–polysaccharide conjugates by FSCE; 67 cm capillary (60 cm to detector)×50 μm I.D.; buffer, 50 m*M* Tris–100 m*M* borate, pH 8.6; voltage, 15 kV; temperature, 20°C; injection, 3.4 mPa for 5 s; final Tt concentration 0.5 mg/ml; UV detection 200 nm. (A) Pn3–Tt, (B) Tt, (C) Pn3–Tt mixed with Tt, (D) Pn4–Tt, (E) Pn4–Tt mixed with Tt.

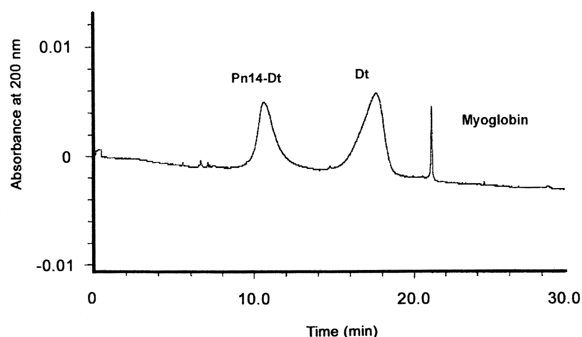

Fig. 2. Separation of Dt and Pn14–Dt by MEKC. Capillary 67 cm (60 cm to detector)×50 μm I.D.; buffer, 25 m*M* SDS–150 m*M* borate, pH≈9.2; voltage, 30 kV; temperature, 20°C; injection, 3.4 mPa for 10 s; UV detection, 200 nm.

serotypes with either Dt or Tt as carrier protein. For example, a mixture of Pn3–Tt and Tt showed almost complete separation by FSCE while a mixture of Pn4–Tt and Tt were overlapping (Fig. 1) (PnX, pneumococcal polysaccharide serotype X; PnX–Tt, pneumococcal polysaccharide serotype X conjugated to tetanus toxoid). Evaluation of MEKC as a more generally applicable method was therefore undertaken. An initial MEKC screening of conjugates prepared from several serotypes appeared promising, so a method was optimized using a mixture of Dt and Pn14–Dt. The conditions determined in this optimization (150 m*M* borate, 25 m*M* SDS at 30 kV, Fig. 2) were then used to survey a variety of conjugates. Figs. 3 and 4 illustrate the electrophoretic behavior of Dt, Tt and several Dt–polysaccharide conjugates. In addition to the examples given, separation has also been obtained for pneumococcal polysaccharide conjugates Pn5–Dt, Pn6B–Dt, and meningococcal polysaccharide–Dt conjugates from serotypes C, Y and W135 (data not shown) as well as MnA–Dt conjugate (see below) (MnX–Dt, meningococcal polysaccharide serotype X conjugated to diphtheria toxoid). These results demonstrate that

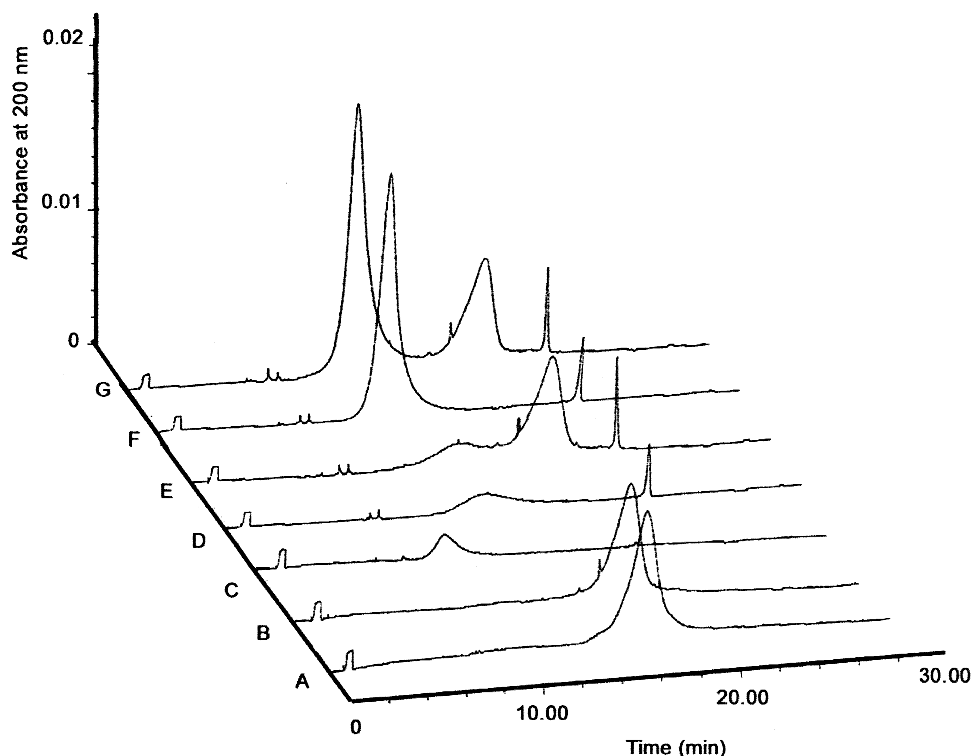

Fig. 3. Separation of Dt, Tt and pneumococcal polysaccharide–Dt conjugates by MEKC. Separation as described in Fig. 2. (A) Tt, (B) Dt, (C) Pn7–Dt, (D) Pn3–Dt mixed with myoglobin, (E) Pn3–Dt mixed with Dt and myoglobin, (F) Pn18C–Dt mixed with myoglobin, (G) Pn18C–Dt mixed with Dt and myoglobin.

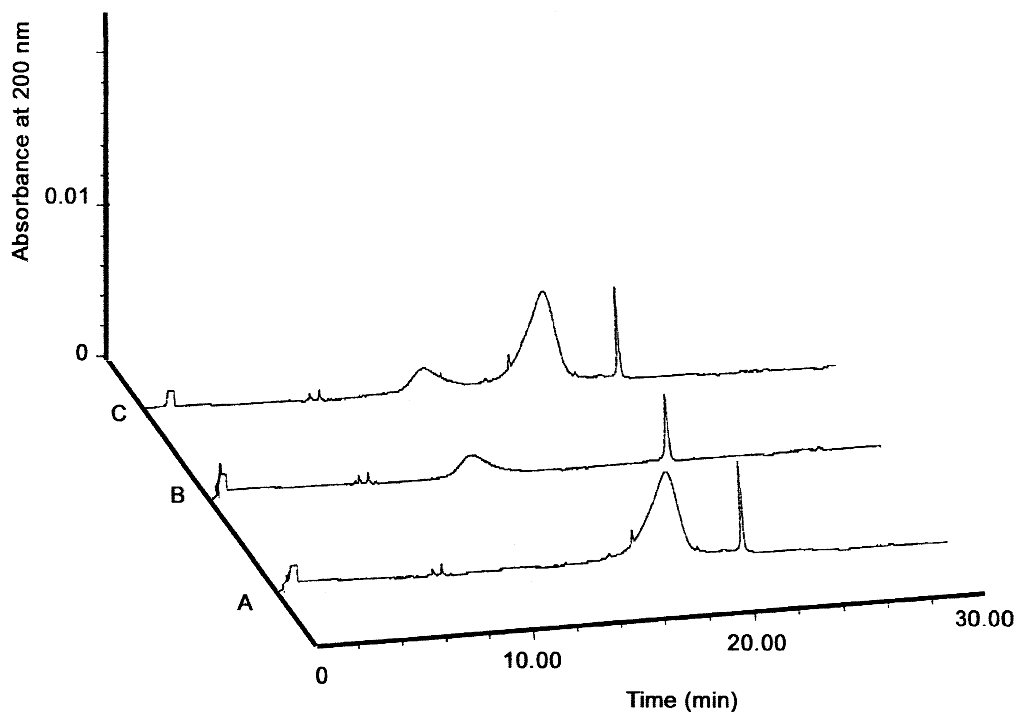

Fig. 4. Separation of Dt, and Pn1–Dt by MEKC. Separation as described in Fig. 2. (A) Dt, (B) Pn1–Dt, (C) Pn1–Dt mixed with Dt. Myoglobin added to all samples.

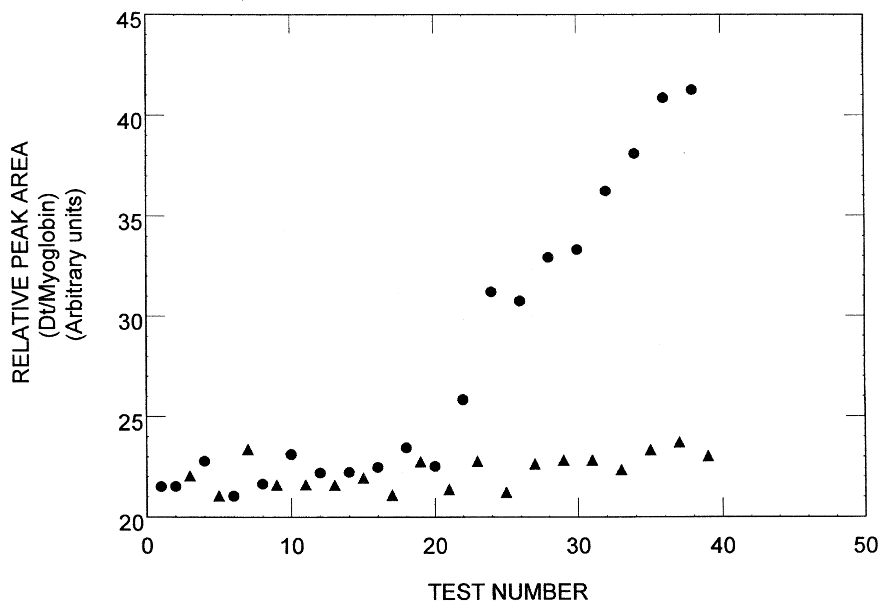

Fig. 5. Repetitive determination of relative Dt peak area. Separation as described in Fig. 2. ●, Single vial sampled repeatedly, ▲, multiple vials sampled two or three times each.

MEKC is widely applicable for separation of toxoid–polysaccharide conjugates prepared from Dt and a variety of polysaccharides.

3.2. Quantitation of Dt

Dt peak area was linear as a function of dilution from ≈2 mg protein/ml to 16 μg/ml and also as a function of sample injection time from 10 to 50 s, the longest injection time tested. In both cases r^2 was >0.99 (data not shown).

Preliminary experiments designed to assess the repeatability of Dt quantitation indicated poor assay precision when absolute peak area was measured. Furthermore, measuring Dt peak area relative to an internal standard (myoglobin) demonstrated good precision for the first 8–10 injections, after which there was an apparent rise in the relative Dt content (data not shown). Repetitive testing was therefore performed using a mixture of Dt and myoglobin that had been divided into several sample vials. One of these vials was sampled repeatedly while each of the remaining vials were sampled only three or four times. Tests of the repeatedly sampled vial and the infrequently sampled vials were interspersed to minimize the impact of possible time-dependent factors such as capillary conditioning and buffer depletion. The results show (Fig. 5) that the

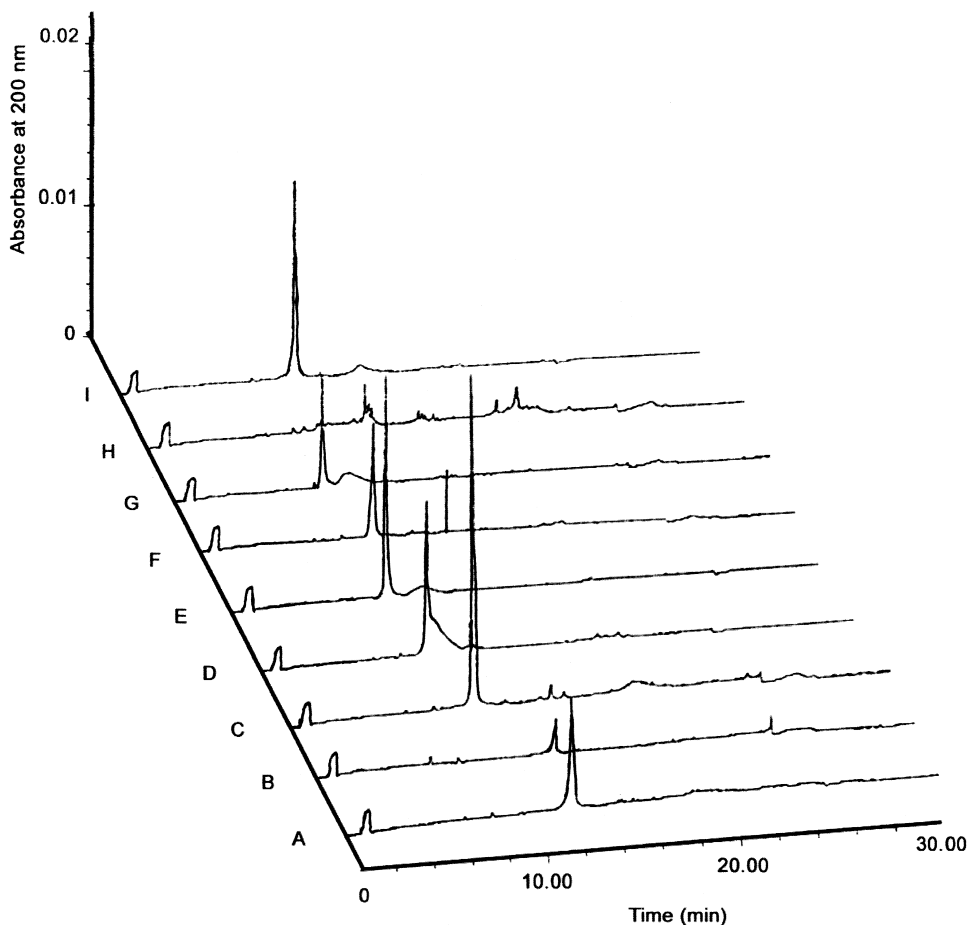

Fig. 6. Testing of pneumococcal polysaccharides by MEKC. Separation as described in Fig. 2. Individual polysaccharides were dissolved in saline to a final concentration of ≈2.0–2.5 mg/ml. (A) Pn1, (B) Pn3, (C) Pn4, (D) Pn5, (E) Pn7F, (F) Pn9V, (G) Pn14, (H) Pn18C, (I) Pn19F. No peaks were observed for serotypes Pn6B and Pn23F (not shown).

repeatedly sampled vial showed an apparent increase in relative Dt peak area beginning after eleven tests. This suggested that repeated sampling introduced a component of the separation buffer that interacted with the protein in the samples. This supposition was confirmed by adding small amounts of 25 m*M* SDS to a mixture of Dt and myoglobin (between 0 and 2 μl/200 μl sample). Increasing amounts of SDS caused an apparent increase in the relative Dt peak area that was similar to the effect noted above (data not shown). Data from the infrequently sampled vials ($n=19$) also show that relative Dt migration time was more precise than absolute migration time (RSD=0.9 and 3.7%, respectively) and that relative Dt peak area was much more precise than absolute peak area (RSD=5.8 and 18.3%, respectively).

3.3. MEKC of polysaccharide

It is reasonable to expect that conditions under which a large amount of free toxoid carrier protein is present (i.e. a failed conjugation reaction) might also result in the presence of free polysaccharide. Eleven different pneumococcal serotypes were therefore tested by MEKC for possible interference in the assay. Fig. 6 shows that no significant overlap occurred between the migration times of Dt or Tt and any of the polysaccharides tested. These results show

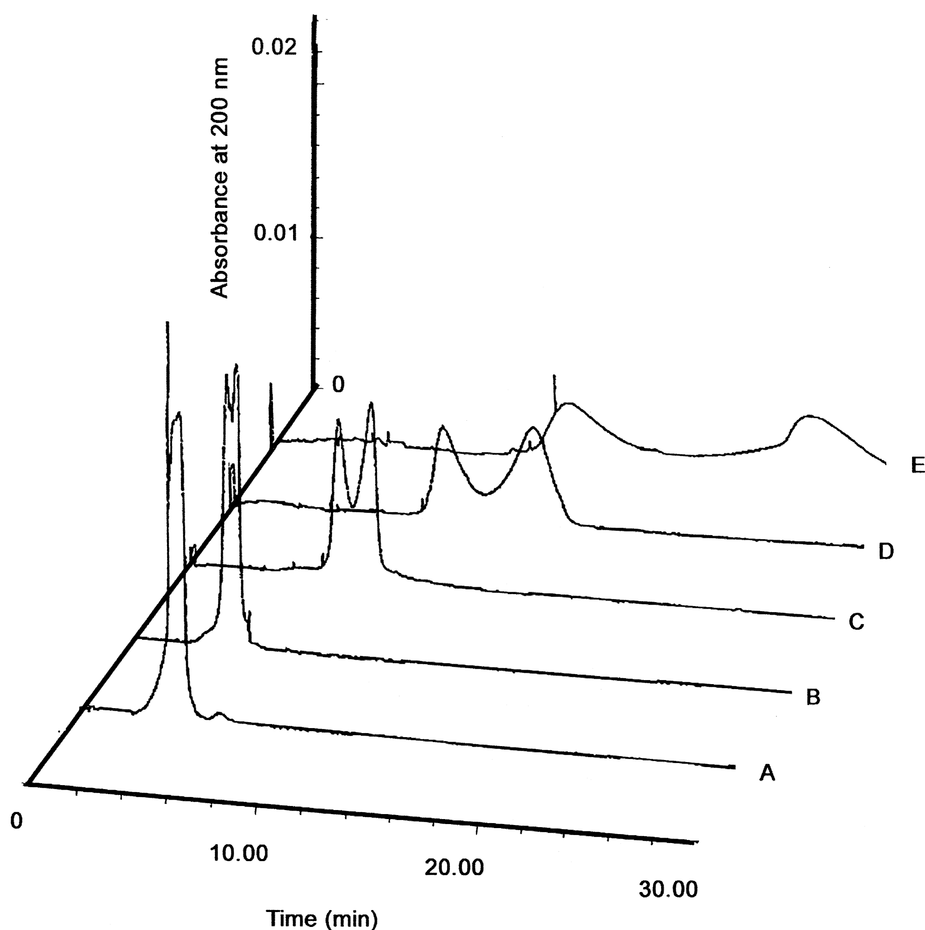

Fig. 7. Separation of Dt and MnA–Dt by MEKC with varying borate concentrations. Separation as described in Fig. 2 except borate concentration was as indicated. (A) 10 m*M*, (B) 50 m*M*, (C) 150 m*M*, (D) 250 m*M*, (E) 350 m*M*.

that the presence of unconjugated polysaccharide would not interfere with determination of Dt or Tt peak area.

3.4. Further optimization

MnA–Dt and Dt is the most poorly resolved mixture tested to date. Several assay parameters were investigated in order to achieve better resolution of these two components. Fig. 7 shows the effect of increasing borate concentration on the separation of MnA–Dt and Dt. Increased levels of borate slowed the migration times of both components, possibly due to decreased electroosmotic flow, and increased their separation. Decreasing the applied voltage also slowed the migration times of the two components, but did not increase separation to the same extent as increasing the borate concentration (data not shown). Increasing the SDS concentration to 50 m*M* (in the presence of 150 m*M* borate) had no effect on assay performance. Some deterioration of the baseline was noted at 100 m*M* SDS (data not shown). We conclude that the assay parameters initially selected are suitable for a wide range of toxoid–polysaccharide conjugates, but experimental conditions may need to be altered in some specific cases.

4. Conclusions

We report the results of our studies on separation of Dt from Dt conjugated to bacterial polysaccharides derived from a number of sources. The method developed is simple to perform, quantitative, widely applicable to vaccines prepared from many different polysaccharides and is reproducible when performed using an internal standard. Myoglobin was demonstrated to be suitable for use as an internal standard. The method may therefore be applicable as a means of monitoring consistency of toxoid incorporation into final product during routine vaccine manufacturing. Our results further suggest that CE may be a useful tool for the study and characterization of protein–polysaccharide conjugate vaccines generally.

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