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α,ω-Bis-quaternary ammonium alkanes as effective buffer additives for enhanced capillary electrophoretic separation of glycoproteins

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Abstract

The egg white glycoprotein, ovalbumin, is known to be microheterogeneous as a result of its varied glycan content. The use of 1,4-diaminobutane (DAB) as a buffer additive has been shown to be key in the high-resolution capillary electrophoretic separation of "glycoforms" of this protein [*Anal. Biochem.* 205 (1992) 115]. Although a separation buffer consisting of 100 m*M* borate and 1 m*M* DAB allowed for adequate separation of ovalbumin glycoforms, prolonged separation times of 35–45 min were undesirable. In the present study, the α,ω-bis-quaternary ammonium alkanes, hexamethonium bromide (C_6MetBr), hexamethonium chloride (C_6MetCl) and decamethonium bromide (C_{10}MetBr) were tested as buffer additives for their effectiveness in the separation of ovalbumin glycoforms. Where 1 m*M* DAB gave optimal separation in *ca.* 45 min, 100 μM C_6MetCl or C_{10}MetBr yielded comparable resolution in less than 20 min. Results with the C_{10}MetBr were better than those obtained with C_6MetBr, indicating that there may be a correlation between effectiveness and alkyl chain length. Use of the chloride salt of C_6Met afforded the same resolution as the bromide salt in slightly shorter analysis time. The rank order for their effectiveness was found to be C_{10}MetBr > C_6MetCl > C_6MetBr > DAB. These results allow for speculation on the mode through which these additives exert their effect on resolution. Included in these are additive-wall coating interactions, protein–additive interactions, protein-wall interactions or any combination of these.

1. Introduction

High-performance capillary electrophoresis (HPCE), a relatively new analytical tool, has been shown to be capable of attaining separation efficiencies superior to those with high-performance liquid chromatography (HPLC). Electrophoretic separation carried out in 75 μm I.D. (or less) capillaries allows for the efficient dissipation of Joule heat and, therefore, non-denaturing electrophoretic separations can be carried out in simple buffers under high fields (up to 30 000 V). HPCE has been shown to be useful for the separation of a diverse array of molecules including ions [1], small organic molecules [2], carbohydrates [3,4], peptides [5] and oligonucleotides [2].

One of the areas where HPCE has had a dramatic impact is with the analysis of glycoproteins. Surface carbohydrates on proteins, once thought to be artifacts, are now known to impart a number of important biological functions including antigenicity, transport, folding and biological activity. HPCE has been shown to be

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useful for resolving the glycoforms of a variety of proteins including ribonuclease [5], recombinant T4 receptor [7], human recombinant erythropoietin [8], leech-derived O-linked glycopeptides [9], and transferrin [10,11].

Determining conditions for the electrophoretic separation of proteins in uncoated fused-silica capillaries has been problematic, mainly due to the inherent nature of proteins for interacting with the capillary wall or other proteins. One of the main ways in which this problem has been circumvented is through the use of buffer additives that either coat the inner wall or augment the buffer to reduce protein–wall interactions [12–16]. We [17] and others [18] have demonstrated the utility of alkyl diamines for the analysis of glycoprotein microheterogeneity. In the present study, a similar class of alkyl compounds, in which the terminal diamino groups have been replaced with quaternary ammonium moieties, is tested. It will be shown that α,ω-bis-quaternary ammonium alkanes not only allow for analysis of microheterogeneity, but are much more efficient than their diamino counterparts.

2. Experimental

2.1. Materials

Sodium hydroxide was purchased from Fisher Scientific. All chemicals for peptide sysnthesis were purchased from Applied Biosystems (Foster City, CA, USA). Borax (sodium tetraborate), boric acid, hexamethonium bromide (C_6MetBr), hexamethonium chloride (C_6MetCl), decamethonium bromide (C_{10}MetBr) and diaminobutane (DAB) were purchased from Sigma (St. Louis, MO, USA). Dimethylformamide (DMF) was purchased from Aldrich (Milwaukee, WI, USA). Ovalbumin was purchased from Pharmacia (Piscataway, NJ, USA) or Sigma.

2.2. Buffer and sample preparation

Borate buffer was made by titrating 25 m*M* sodium tetraborate with 100 m*M* boric acid until the desired pH was obtained. For use in the separation buffer (100 m*M* borate), C_6MetBr and other additives were diluted from a stock solution (1 *M*, in Milli-Q purified water) to the desired concentration. All buffers were made with Milli-Q (Millipore) water, and filtered through an 0.2-μm filter (Gelman) before use. Proteins were dissolved in the running buffer without modifier and filtered through an 0.22-μm micro-centrifuge filter (Millipore).

2.3. Instrumentation

HPCE was carried out on a Beckman P/ACE System 2100 interfaced with an IBM 55SX computer utilizing System Gold software (version 7.1) for instrument control and data collection. All peak information (migration time) was obtained through System Gold software.

Reversed-phase (RP) HPLC of 25 μg of ovalbumin was carried out on an ABI 130A microseparation system with an ABI RP-300 C_8 reverse-phase column (100 × 2.1 mm) (Applied Biosystems).

2.4. HPCE separation conditions

Capillaries were bare fused-silica (Polymicro Technologies, Phoenix, AZ, USA) of 87 cm (80 cm to the detector) × 375 μm O.D. × 50 μm I.D. For analyses, the following method was typically used: a three column-volume rinse with separation buffer, 3-s pressure injection of a protein solution in separation buffer or water (3.3 nl; 0.5–1.5 mg/ml), followed by a 3-s pressure injection of the neutral marker, DMF (1:5000 in water); separation at 25 kV (constant voltage with the inlet as the anode and the outlet as the cathode), a three column-volume wash with 0.1 *M* NaOH followed by a three column-volume rinse with separation buffer. All electrophoretic separations were carried out at constant voltage (typically 25 kV) and capillary temperature was maintained at 28°C. Detection was by absorbance at 200 nm.

3. Results and discussion

Ovalbumin is a glycoprotein found in avian egg white and has a M_r of ca. 43 000. It has two

potential asparagine-linked glycosylation sites, one on amino acid 292 and the other on 312 (total of 385 amino acids). Studies have demonstrated that only the Asn_{292} is glycosylated *in vivo* [19] and that Asn_{312} can be glycosylated *in vitro* [20]. As with the carbohydrate component of many glycoproteins, the glycan structure of ovalbumin is heterogeneous [21]. A total of nine different oligosaccharide chains have been identified [22–26], all of which have been classified as high-mannose or hybrid structures. The hybrid and high-mannose forms are present at approximately a 1:1 ratio.

The RP-HPLC separation of ovalbumin on a C_8 column is shown in Fig. 1A. The resolution is relatively poor and the presence of multiple broad peaks is suggestive of glycoprotein heterogeneity. The profile in Fig. 1B represents the electrophoretic separation of the same ovalbumin solution in an 87 cm capillary (effective length of 80 cm) in 100 m*M* borate, pH 8.4. While the analysis time is decreased significantly in comparison with RP-HPLC, the resolution has only been moderately enhanced. Electrophoretic separation of the same sample in the same capillary with borate buffer containing 1 m*M* DAB results in the resolution of the multitude of components present in the sample (Fig. 1C). Decreasing the DAB concentration below 1 m*M* has been shown to lead to loss of resolution [17]. Concomitant with the enhanced resolution is a decrease in electroosmotic flow (EOF), consistent with observations reported in earlier studies [17,27]. It is reasoned that the enhanced resolution results from an increase in analyte residence time brought about by the combination of decreased EOF (from addition of 1 m*M* DAB) and a longer than normal effective capillary length (80 cm *vs.* 40–50 cm capillaries typically used in HPCE) [17]. The type of buffer used also appeared to play a key role in the resolution. Although the details of the mechanism are not completely understood, DAB has been postulated to exert its effect on the separation through a masking of silanol groups. This affects the ζ potential and, subsequently, the EOF.

In a study aimed at understanding the mechanism through which α,ω-diaminoalkanes like DAB function, both the bifunctional character

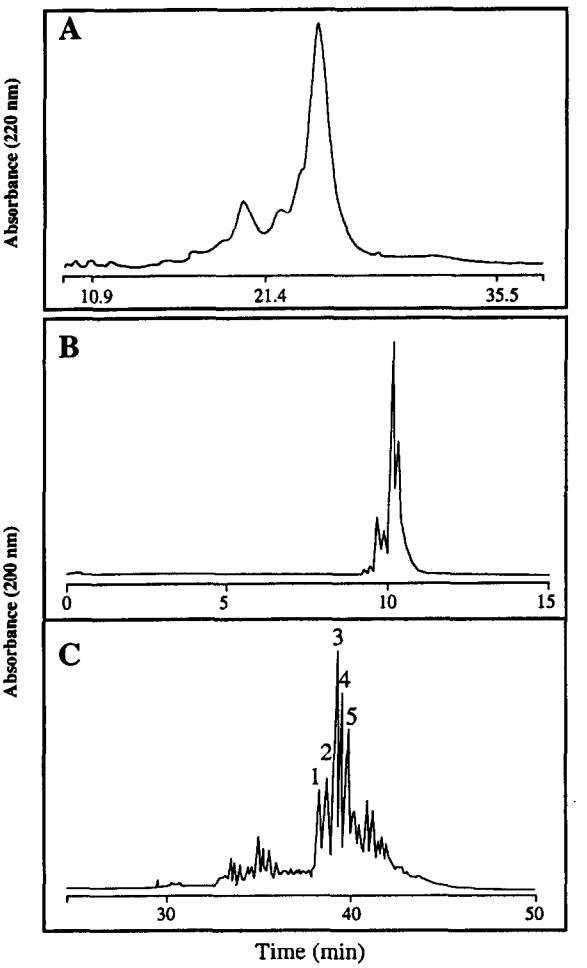
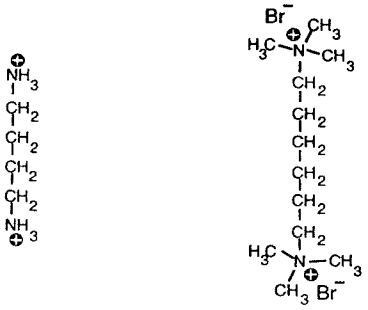


Fig. 1. The effectiveness of DAB for the resolution of ovalbumin isoforms. (A) RP-HPLC of ovalbumin (1 mg/ml; 25-μl injection). Column: ABI RP-300 (C_8, 100 × 2.1 mm). Buffers: A = 5% acetonitrile in water, 0.1% trifluoroacetic acid (TFA); B = 70% acetonitrile in water, 0.1% TFA. Flow-rate: 0.2 ml/min with a gradient of 50% to 100% B over 50 min. (B) Capillary electrophoresis of ovalbumin [1 mg/ml, 3-s pressure (0.5 p.s.i., 1 p.s.i. = 6894.76 Pa) injection]. Buffer: 100 m*M* borate, pH 8.4, no additives. (C) Capillary electrophoresis of ovalbumin [1 mg/ml, 3-s pressure (0.5 p.s.i.) injection]. Buffer: 100 m*M* borate, pH 8.4, 1 m*M* DAB. Capillary: bare silica, 87 cm (80 cm to the detector) × 50 μm I.D. Separation: 25 kV (17 μA); 28°C.

and the length of the alkyl chain were found to play a role in their effectiveness for glycoform resolution [6]. Furthermore, there was an excellent correlation between those alkyl diamines which effected ovalbumin resolution and those which optimally affected EOF. It was, therefore,



1,4-Diaminobutane Hexamethonium bromide

Fig. 2. Structures of DAB and C_6MetBr.

of interest to determine whether similar compounds containing α,ω-quaternary ammonium groups (*vs.* protonatable amines) were capable of similar effects. Fig. 2 illustrates the structure of the two classes of compounds, highlighting the differences in charge at the termini of each molecule. In theory, quaternary ammonium compounds are advantageous over the α,ω-diaminoalkanes as a result of two properties: their ionization state is not pH-dependent and addition does not alter the buffer pH.

The electropherograms in Fig. 3 show the effect on ovalbumin resolution of hexamethonium bromide (C_6MetBr) added to 100 m*M* borate buffer, pH 8.3 (micromolar values on the *z*-axis represent the final concentration in the separation buffer). Under these conditions, baseline resolution of all peaks could be achieved with 1 m*M* C_6MetBr at the expense of undesireably long analysis times (> 60 min). The optimal concentration for adequate resolution within a reasonable analysis time-frame was 300 μM C_6MetBr (25–35 min). These results were somewhat surprising since faster separation was attained with an additive concentration 3-fold

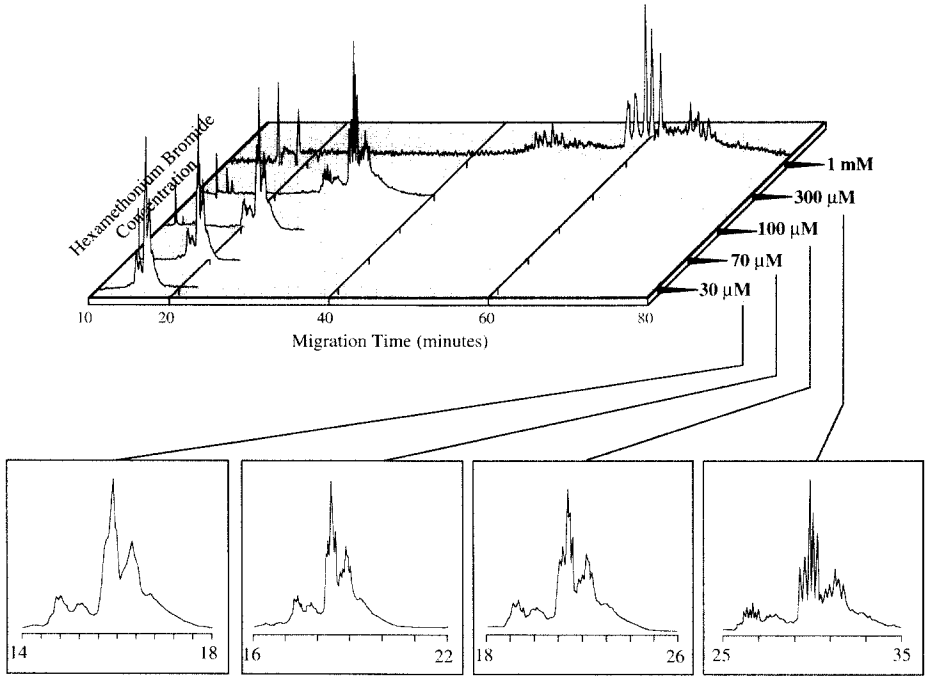


Fig. 3. The dependence of ovalbumin resolution on hexamethonium bromide concentration in the separation buffer. The top part shows the separations on the same time scale. The lower panels, which are expanded time scales (min) profiles of the upper traces, depict the enhanced resolution with increasing concentrations of hexamethonium bromide. From left to right, 30, 70, 100 and 300 μM C_6MetBr final concentration in 100 m*M* borate, pH 8.4. Sample: ovalbumin [1 mg/ml, 3-s pressure (0.5 p.s.i.) injection]. Capillary: bare silica, 87 cm (80 cm to the detector) × 50 μm I.D. Separation: 25 kV (17 μA); 28°C.

lower than DAB. Reproducibility for replicate analyses with 300 μM C_6MetBr in the separation buffer ($n = 3$) was found to be 0.53, 0.53, 0.54, 0.54 and 0.54% R.S.D. for peaks 1–5 (as labelled in Fig. 1C), respectively. The level of reproducibility and resolution associated with C_6MetBr addition to the separation buffer make it ideal for preparative-scale analyses. This contrasts with the use of higher concentrations of DAB (*e.g.*, 3–5 mM) where baseline resolution was associated with significant band broadening and extremely lengthy migration times [17].

The importance of the counterion in the overall separation was evaluated by comparing separations carried in the presence of C_6MetCl and C_6MetBr (Fig. 4). It was interesting to find that the dependence on concentration with C_6MetCl was significantly different than that with C_6MetBr. One significant difference is the fact that adequate separation is attained with 100 μM, a concentration 3-fold lower than that required with C_6MetBr and 10-fold lower than that required with DAB. More important is that adequate resolution is attained in less than 20 min (in comparison with 300 μM C_6MetBr in 33 min and 1 mM DAB in 40 min). This is suggestive of the chloride salt being more effective than bromide salt and may be the result of the higher mobility of chloride as a counterion.

To determine whether the α,ω-quaternary ammonium compounds shared some of the chain length-dependent structure–activity relationships observed with the α,ω-diaminoalkanes [6], C_{10}MetBr was tested for its ability to effect ovalbumin separation (Fig. 5). Similar to the C_6MetCl, resolution of the ovalbumin isoforms was achieved with a concentration of 100 μM C_{10}MetBr. Adequate resolution is attained in

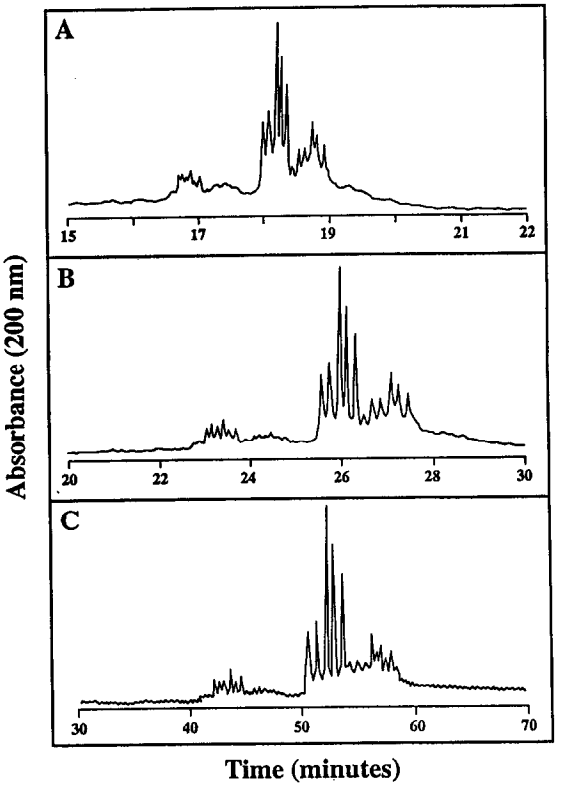


Fig. 4. The dependence of ovalbumin separation on C_6MetCl concentration. (A) 100 μM, (B) 300 μM and (C) 1 mM C_6MetCl. Sample and other conditions as in Fig. 3.

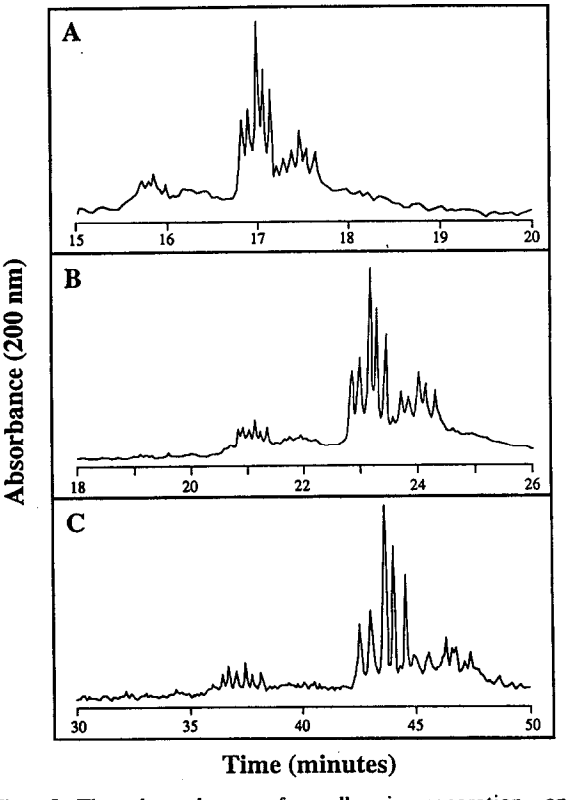


Fig. 5. The dependence of ovalbumin separation on C_{10}MetBr concentration. (A) 100 μM, (B) 300 μM and (C) 1 mM C_{10}MetBr. Sample and other conditions as in Fig. 3.

slightly less time (18 min) than observed with C_6MetCl (20 min) with a reproducibility ($n = 3$) of 0.37, 0.37, 0.38, 0.38 and 0.38% R.S.D. for peaks 1–5, respectively. Comparison of the migration times obtained with a 300 μM concentration of each of the additives in separation buffer shows the order of effectiveness to be C_{10}MetBr > C_6MetCl > C_6MetBr (Fig. 6). This suggests that the alkyl chain length may be a more important parameter than the counterion in effecting resolution.

Based on the markedly shorter analysis times required for resolution of the isoforms in the presence of C_{10}MetBr (18 min) and C_6MetBr (33 min), it was assumed that these additives would have less effect on the slowing of EOF than that observed with DAB (45 min). EOF was monitored in each analysis by the co-injection of DMF with the sample. Surprisingly, the effect of C_6MetBr and C_{10}MetBr on the slowing of EOF was found to be similar to that of DAB (Fig. 7). This indicates that, despite a significant repression of EOF, quaternary ammonium alkane additives allow for ovalbumin glycoforms to be resolved in less than half the analysis time (18 min with C_{10}MetBr *vs.* 40 min with DAB). This is suggestive that alteration in EOF is *not* the only parameter involved in the additive-induced enhancement of resolution.

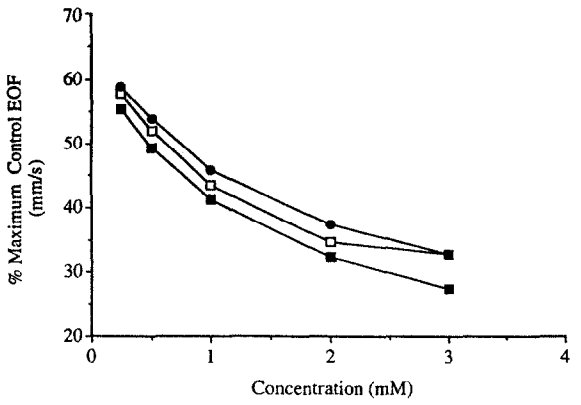


Fig. 7. The effect of DAB (□), C_6MetBr (■) and C_{10}MetBr (●) on EOF. The two amines were added to 100 mM borate buffer, pH 8.4 at the indicated final concentrations. The effect on EOF was determined by the migration time of a neutral marker, DMF. Capillary: bare silica, 87 cm (80 cm to the detector) × 50 μm I.D. Separation: 25 kV (17 μA); 28°C.

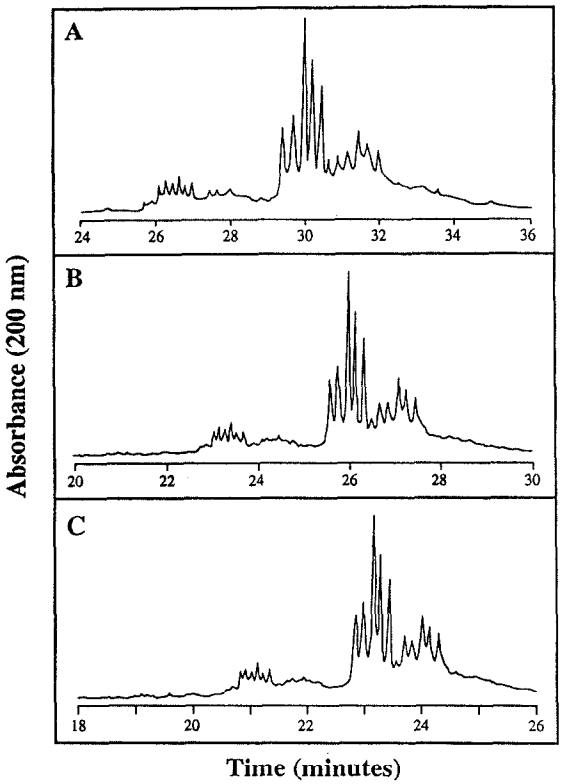


Fig. 6. A comparison of effect of 300 μM concentration of each of the bis-quaternary ammonium additives on ovalbumin resolution. (A) 300 μM C_6MetBr, (B) 300 μM C_6MetCl, (C) 300 μM C_{10}MetBr. Sample and other conditions as in Fig. 3.

As a final step in the evaluation of the quaternary ammonium compounds as additives, we determined whether they could effect the separation of other microheterogenous glycoproteins. Fig. 8 shows the resolution of the isoforms of human chorionic gonadotropin (hCG) with 1 mM hexamethonium bromide and 25 mM borate buffer, pH 8.4. Seven of the eight isoforms are clearly resolved. This presents the possibility that bis-quaternary ammonium alkyl compounds may be effective buffer additives for the resolution of isoforms of other glycoproteins.

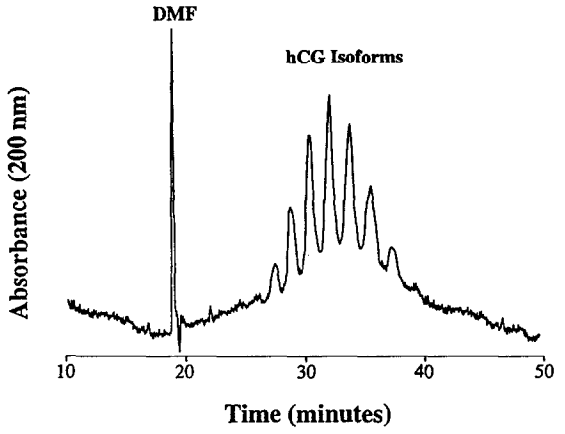


Fig. 8. Effect of hexamethonium bromide on the resolution of hCG isoforms. Human chorionic gonadotropin [5 mg/ml, 5-s pressure (0.5 p.s.i.) injection]. Buffer: 25 m*M* borate, pH 8.4 containing 1 m*M* hexamethonium bromide. Capillary: bare silica, 87 cm (80 cm to the detector) × 50 μm I.D. Separation: 25 kV (8.5 μA); 28°C.

4. Conclusions

This study has demonstrated that bis-quaternary ammonium alkyl compounds appear to be more effective at resolving glycoprotein isoforms than their alkyl diamine counterparts. This stems not only from the fact that resolution is obtained at lower concentrations, but that adequate resolution is obtained in dramatically shorter analysis times. The observed order of effectiveness (C_{10}MetBr > C_6MetCl > C_6MetBr > DAB) indicates that the ideal quaternary ammonium additive possesses a long alkyl chain (C_{10}) and has chloride as a counterion (*i.e.*, decamethonium chloride). From a mechanistic perspective, the fact that similar resolution from two different additives with a greater than 2-fold difference in analysis time and minimal differences in EOF is suggestive that the magnitude of EOF is not the only factor leading to the enhanced resolution. One likely difference between the alkylamines and the quaternary ammonium-type compounds is that the latter tend to be better "ion exchangers". The fact that both classes of amines have a tendency for wall (silanol) interaction suggests that additional parameters, such as the interaction of the additive with protein analytes or interaction of the protein with wall-bound additive (chromatography?) should be considered.

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