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Characterization of sulphoalkyl ether derivatives of β-cyclodextrin by capillary electrophoresis with indirect UV detection

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Abstract: A capillary electrophoresis method which characterizes the degrees of substitution of heterogeneous sulphoalkyl ether β-cyclodextrin derivatives is described. The separation is based on the different electrophoretic mobilities observed from changes in the overall charge of the molecule as a result of substitution. Individual peaks of the electropherogram then provide a measure of each degree of substitution of the parent β-cyclodextrin. Detection of these β-cyclodextrin derivatives is performed by indirect UV detection.

Keywords: *Capillary electrophoresis; sulphoalkyl ether β-cyclodextrin(s); β-cyclodextrin; indirect UV detection.*

Introduction

β-cyclodextrin (β-CD) is a naturally occurring cyclic oligosaccharide consisting of seven α-1,4-linked glucose units. Because of the conformational constraints of the cyclic structure the molecule forms a conical shape, with a hydrophilic outside edge and an internal hydrophobic cavity. The primary hydroxyls of each glucose monomer are oriented towards the narrow end of the cavity, while the equatorially constrained secondary hydroxyls form the rim of the wide end of the cavity. Consequently, each end of the cone is termed either the 'primary face' or 'secondary face' of the molecule. This molecular structure is conducive to the formation of inclusion complexes with a variety of chemical substances [1–5]. Upon inclusion with the host cyclodextrin, the micro-chemical environment of the included substance may change. The nature of the inclusion is often very specific, for example, one enantiomer of a racemic mixture may have a greater affinity for the host cavity. Both pharmaceutical scientists and analytical chemists have exploited these properties for; the aqueous solubility enhancement of poorly soluble drugs, stability enhancement, solvent protection, controlled release drug delivery, taste masking of unpalatable drugs, the optical resolution of racemic drug mixtures, and the augmentation of analytical detection from fluorescence or ultraviolet enhancement or suppression [1–13].

Unfortunately, the clinical use of β-CD as a pharmaceutical excipient is limited by both haemolysis and nephrotoxicity observed with chronic systemic administration [14–16]. The nephrotoxicity appears to be related to the relatively low aqueous solubility of β-CD. It is believed that β-CD accumulates and eventually precipitates the renal epithelium, eventually resulting in necrosis [14, 15]. Theoretically, derivatives of β-CD with improved aqueous solubility should exhibit less nephrotoxicity due to decreased nephro-tubule reabsorption.

A series of sulphoalkyl ether derivatives of β-CD have been produced in our laboratories with the express purpose of improving the toxicity, aqueous solubility and subsequent pharmaceutical efficacy of β-CD [17]. These sulphoalkyl ether derivatives of β-CD offer distinct advantages over other β-CD derivatives [1, 4, 5, 18, 19] designed to improve aqueous solubility. Many of these other β-CD derivatives have either bulky and/or charged substituents (e.g. sulphates) very close to the 'primary' or 'secondary' face of the molecule which could alter the inclusion properties

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dramatically, or simple alkylation (e.g. permethyls) of the hydroxyls, which does not interfere with the inclusion properties and in fact may improve the inclusion of some drugs, yet, results in only a marginal improvement in solubility. With these sulphoalkyl ether derivatives of β-CD the sulphonate group is spaced from the cyclodextrin cavity with an alkyl side chain to ensure the new substituents do not interfere with the inclusion process. It has subsequently been found that these derivatives have much greater aqueous solubility than β-CD, yet retain much the same inclusion properties [20]. Since the anionic moiety is a sulphonate, the physicochemical properties (including solubility) of the molecule are relatively independent of solution hydrogen ion concentration. While a calculation of a theoretical average degree of substitution is possible from the molar ratio of the reactants, the resulting derivatized material is heterogeneous, consisting of a mixture of isomers with varying degrees of substitution. As a consequence of this, a simple rapid method for the characterization of these derivatives was developed. Complete description of the material is further complicated by the number of positional isomers possible within each degree of substitution, each glucose unit of β-CD having three points of substitution at the hydroxyl groups in the 2, 3 and 6 positions. The method described in this paper characterizes the material in terms of the varying degrees of substitution, no attempt is made to differentiate the positional isomers within each degree of substitution.

Attempts by this laboratory to develop an HPLC method for the separation of the heterogeneous mixture into fractions representative of each degree of substitution has proved unsuccessful. The large polarity of the material over all pH ranges precludes the use of hydrophobic stationary phase systems such as a reverse phase column, and its polyanionic nature proved problematic for both weak and strong anion exchange chromatography with either poor resolution of fractions with low levels of substitution or complete retention of fractions with high levels of substitution. Nuclear magnetic resonance techniques were complicated by the multitude of positional isomers for each substitution, and mass spectrometry techniques were complicated by isotopic contributions particularly at higher substitutions. In contrast, separation of various degrees of sulphoalkyl ether substitution of β-CD was achieved by capillary electrophoresis. For this analysis, the high efficiency and speed of capillary electrophoresis appears to offer a distinct advantage over other separation techniques [21]. As the cyclodextrin derivatives have no suitable chromophore or fluorophore, indirect UV detection was used [22, 23]. The indirect UV detection was based upon the hypsochromic shift observed with inclusion of benzoic acid as the benzoate anion into the β-CD cavity [24]. This approach provided complete resolution of mono- to deca-derivatized β-CD and allowed characterization of derivatized mixtures.

Materials and Methods

Chemicals

All chemicals used were of at least analytical reagent grade purity. β-CD was purchased from Amaizo (Hammond, IN, USA). Benzoic acid and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All solutions were prepared in doubly distilled water.

Preparation of sulphoalkyl ether derivatives of β-cyclodextrin

Sulphoalkyl ether derivatives of β-CD were prepared according to the procedure of Rajewski and Stella [17]. One mole equivalent of β-CD was added to 14 mole equivalents of aqueous sodium hydroxide and allowed to dissolve. Five mole equivalents of the appropriate alkyl sultone was then added and the reaction was allowed to proceed for 24 h at 70 ± 3°C, with continuous stirring. The average degree of substitution could be pre-determined by the molar ratio of β-CD and alkyl sultone. The reaction mixture was then allowed to cool to room temperature, diluted with doubly distilled water, neutralized with hydrochloric acid, and filtered. The filtrate was then concentrated by ultrafiltration. Any unreacted β-CD was removed by anion exchange chromatography. Subsequent dialysis removed any residual sodium chloride.

This procedure was used to prepare sulphobutyl ether β-CD derivatives with average degrees of substitution of 4 and 7 [β-CD-SBE (4) and β-CD-SBE (7)], and sulphopropyl ether derivatives with an average degree of substitution of 4[β-CD-SPE (4)]. An attempt was made to prepare pure fractions of mono

substituted sulphobutyl ether β-CD [β-CD-SBE (1)] and bis substituted sulphobutyl ether β-CD [β-CD-SBE (2)]. These fractions were separated from higher degrees of substitution of β-CD-SBE (4) by means of anion exchange chromatography as described by Rajewski [20].

Average degrees of substitution of the final product was confirmed by elemental analysis and NMR [17, 20]. Patterns of composition, including the purity of β-CD-SBE (1) and β-CD-SBE (2), were determined with the technique described in this paper. Residual β-CD content was found to be less than 2% (w/w) for all the products [R.J. Tait, N.C. Chetwyn, D.O. Thompson and V.J. Stella, in preparation].

Electrophoretic apparatus

Each end of a 60 cm × 0.050 mm i.d. uncoated fused silica capillary purchased from PolyMicro Technologies Inc. (Phoenix, AZ, USA) was placed in 10 ml contact solutions of running electrolyte, within which was also placed the platinum wire electrodes. The meniscus of the contact solutions was maintained at the same height to minimize flow of solution within the capillary due to gravity. A Spellman Model CZE1000R high voltage power supply (Plainview, NY, USA) maintained the potential difference between the electrodes. A Plexiglas enclosure with an interlock system protected the operator. The on-column detection window was positioned in the cell holder of the ISCO Model CV4 UV–vis detector (Lincoln, NE, USA) 30 cm from the injection end of the capillary. The detection window was prepared by burning the polyimide coating and then wiping off the exposed fused silica with acetone.

Samples were introduced onto the capillary either by electrokinetic migration or pressure injection. All electrokinetic migration injections were performed at 5 kV for 30 s. Pressure injections were performed at 5 psi for 2 s. The pressure was maintained with GenEx ultra high purity nitrogen gas (Des Moines, ID, USA) actuated with a Mac 200 series pneumatic valve (Wixom, MI, USA) and time controlled by a Potter and Brumfield CNT series electronic relay (Princeton, IN, USA). The capillaries were treated with at least five volumes of 0.1 M sodium hydroxide at the start of each day, and were flushed with at least five volumes of eluant buffer between each electrophoretic run. The running electrolyte consisted of 30 mM benzoic acid adjusted to pH 6.0 with 0.1 M TRIS. The detection wavelength was 254 nm.

UV absorbance spectroscopy

Absorption spectra were recorded for various molar ratios of benzoic acid and β-CD-SBE (1) on a Perkin–Elmer Lambda 6 UV–vis spectrophotometer (Norwalk, CT, USA). Solution pH was adjusted to 6.0 with the addition of 0.1 M TRIS base. A path length of 1 mm and slit width of 1 nm was used for all spectra.

Results and Discussion

Spectroscopy of included benzoic acid

As β-CD and its derivatives show virtually no absorbance in the UV range, an indirect UV detection method was used [22, 23]. In an attempt to better understand the principle of detection, a number of spectra of 10 mM benzoic acid with various concentrations of β-CD-SBE (1) were compared (Fig. 1). β-CD-SBE (1) was used because it had far greater solubility than β-CD and it represented a homogeneous fraction of the sulphoalkyl ether derivatives. In the presence of β-CD-SBE (1), a hypsochromic shift of the benzoic acid spectrum is observed. The size of the hypsochromic shift increased with increased β-CD-SBE (1) concentration. The shift of the UV absorbance spectrum of benzoic acid in the presence of cyclodextrins has been attributed to changes to the micro-chemical environment of benzoic acid with inclusion into the cyclodextrin cavity, where intermolecular hydrogen bonding occurs [25, 26]. The results obtained here suggest that the detection of β-CD and derivatives of β-CD by indirect UV detection with benzoic acid as the absorbing species involve a reduced absorbance as a consequence of the shift to the absorbance spectra upon inclusion.

Pressure versus electrokinetic injection

Figure 2 compares the response factors obtained for β-CD-SBE (1) when the sample was introduced by electrokinetic injection method or pressure injection method. Linearity is observed over the concentration range studied for β-CD with both the pressure and electrokinetic injection methods (not shown in Fig. 2; respective correlation coef-

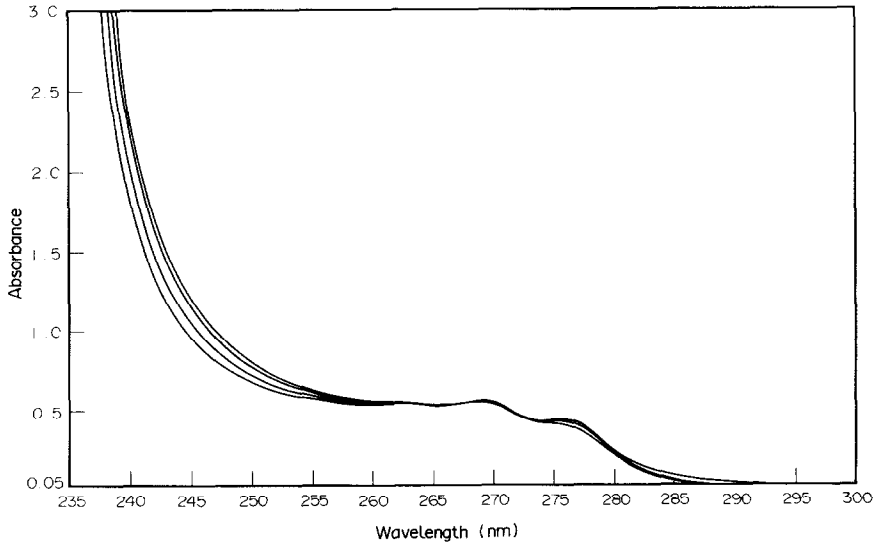

Figure 1
UV absorbance spectra of 10 mM benzoic acid (pH 6.0) in various concentrations of β-CD-SBE (1). Curves indicating progressively increased hypsochromic shift, are due to the presence of 0, 20, 50 and 100 mM concentrations of β-CD-SBE (1).

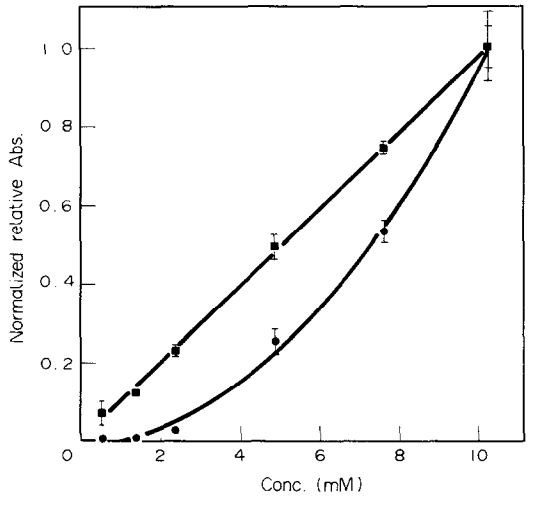

Figure 2
Plots of relative response versus concentration for β-CD-SBE (1) introduced by electrokinetic (●) and pressure (■) injection. Electrokinetic injections were performed at 5 kV for 30 s and pressure injections at 5 psi for 1 s. The relative absorbance has been normalized with respect to the high concentration sample for each injection method. The field strength was maintained at 250 V cm^{-1}.

ficients 0.9990 and 0.9970, over the concentration range 10–0.1 mM, with the largest relative standard deviation being 5.3%), and for the β-CD-SBE (1) with the pressure injection method. However, significant deviation from linearity is observed for the β-CD-SBE (1) with the electrokinetic injection method. The shape of the curve suggests that the introduction of analyte into the capillary was inhibited, particularly at low concentrations. These observations are consistent with the bias that has previously been reported for electrokinetic injection of solutions with high electrical resistance [27]. Clearly, the electric field used to promote the flow of the analyte solution into the capillary with electrokinetic injection, discriminates against anionic derivatized β-CDs, as these anionic species migrate towards the anode and away from the capillary opening during the injection period [28, 29]. This phenomenon can be overcome by preparing the analyte to be introduced into the capillary in solutions of relatively high ionic strength [27–29]. If the ionic strength of the injection solution is sufficiently high to be independent of the analyte concentration then migration effects and electroosmotic effects due to solution resistance are eliminated. The need to prepare analyte in buffer solutions further complicates the injection procedure, consequently the pressure injection method was used for all subsequent experiments.

Response factors for degrees of substitution

Full characterization of derivatized β-CD consisting of a mixture of various degrees of substitution would require quantitation of each peak representing each degree of substitution within the mixture. Ideally, this would involve

comparisons with pure standards of known composition. Unfortunately, standards of known composition were only available for β-CD, β-CD-SBE (1) and β-CD-SBE (2). Linearity was observed for each compound over the concentration range studied. However, the response factor (as determined from relative peak areas) was observed to increase as the degree of substitution increased. This indicates that the detection response is dependent on the degree of substitution, making comparisons between each peak representing different degrees of substitution difficult.

The change in response observed with different degrees of substitution does not appear to be a function of the detection system as the extent of the hypsochromic shift of the benzoic acid spectra was observed to be the same with β-CD-SBE (4) and β-CD-SBE (1). As pressure injection was used, the amount of material introduced into the capillary should be independent of the degree of substitution. It is most likely that the enhanced response is associated with changes to the effective velocity for electrophoretic fraction representing each degree of substitution [30, 31]. The area of each peak is measured as the response as a function of the residence time of the peak in the absorbance window of the spectrophotometer. The effective velocity of each electrophoretic fraction is a function of the electroosmotic flow of solution through the capillary and the electrophoretic mobility of the fraction. Therefore, fractions representative of higher degrees of substitution will have greater electrophoretic mobilities against the electroosmotic flow, effectively slowing the movement of the fraction, which in turn increases the residence time in the absorbance window of the spectrophotometer and increases the area of the peak that results. To overcome this problem a detection system which provides response factors independent of the degree of substitution would be required. The use of response factors from either β-CD, β-CD-SBE (1) or β-CD-SBE (2) for any of the peaks representing higher degrees of substitution would result in an overestimation of the amount of material.

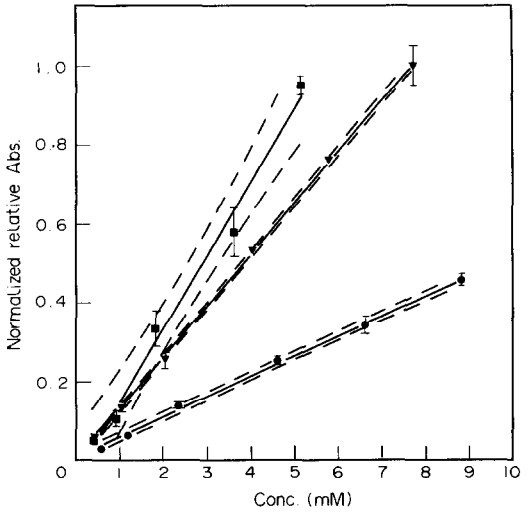

Figure 3
Standard curves for β-CD (●), β-CD-SBE (1) (■) and β-CD-SBE (2) (▲) introduced by pressure injection at 5 psi for 1 s. The relative absorbance for each curve was normalized with respect to the high concentration sample for β-CD-SBE (1). The 95% confidence intervals are indicated by the dashed lines. The respective response factors are: 0.0512, 0.1304 and 0.1850 mM^{-1} and correlation coefficients are: 0.9990, 0.9998 and 0.9947. The field strength was maintained at 250 V cm^{-1}.

Characterization of material

A series of derivatized β-CDs was characterized by means of the capillary electrophoresis system. As described previously, each derivative was distinguished by either the type of substituent or the average degree of substitution. Optimal separation of the β-CD-SBEs and β-CD-SPEs was achieved with an applied potential of 417 V cm^{-1} and 333 V cm^{-1}, respectively. At field strengths below these levels peak shapes deteriorated such that each fraction representative of each degree of substitution consisted of multiple overlapping peaks. Good detection limits were achieved with benzoic acid concentrations between 10 and 40 mM, and was optimal at 30 mM. Detection limit and migration time was independent of pH above pH 5.0.

Figure 4(a)–(e) shows examples of electropherograms of β-CD, β-CD-SBE (1), β-CD-SBE (2), β-CD-SBE (4) and β-CD-SBE (7), respectively. The electropherogram of β-CD-SBE (1) and β-CD-SBE (2) clearly show that in each case only one electrophoretic fraction exists, with no poly substituted fractions present. In contrast, the electropherograms of β-CD-SBE (4) and β-CD-SBE (7) with average degrees of substitution of four and seven and that of Fig. 5 representing β-CD-SPE (4) with an average degree of substitution of four show the materials to be a mixture of derivatives with many degrees of substitution present. Peak assignments were made based upon retention time comparisons with β-CD,

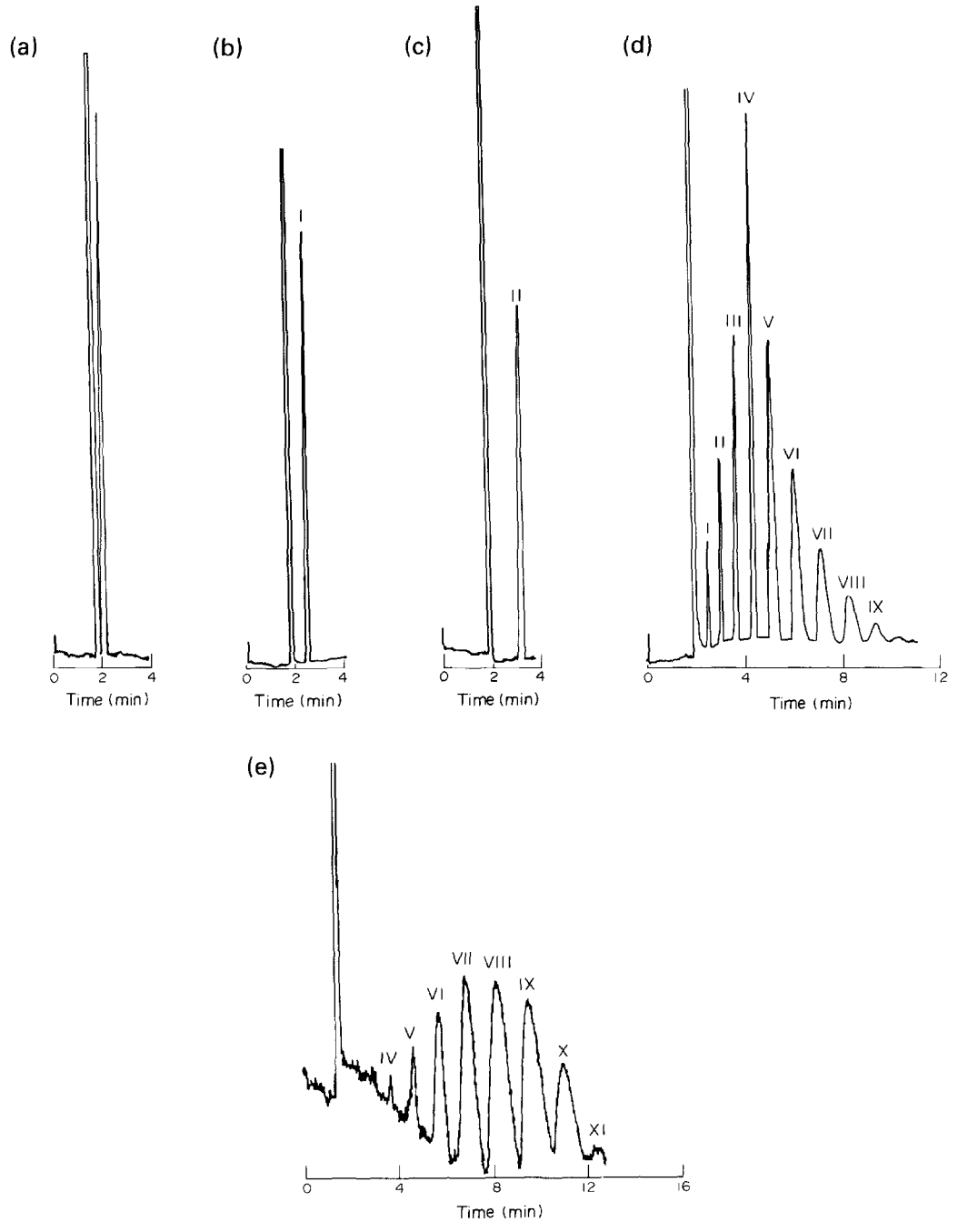

Figure 4
Electropherograms of (a) 8.8 mM β-CD, (b) 7.7 mM β-CD-SBE (1), (c) 6.7 mM β-CD-SBE (2), (d) 5.6 mM β-CD-SBE (4) and (e) 4.5 mM β-CD-SBE (7). Each sample was introduced by pressure injection at 5 psi for 1 s. The field strength was maintained at 417 V cm^{-1}. The roman numerals above each peak indicate the degree of substitution the peak represents. The response was measured as a *loss* of absorbance.

β-CD-SBE (1) and β-CD-SBE (2). It is believed that each peak of the electropherogram represents separated fractions with a specific degree of substitution, the greater the degree of substitution the later eluting the fraction. For example, the electropherogram for β-CD-SBE (4) shows seven peaks representing specific degrees of substitution of one to seven. A similar pattern of composition occurs with a bell type distribution of the peaks centered about the largest peak. In each case, the largest peak matched the average degree of sub-

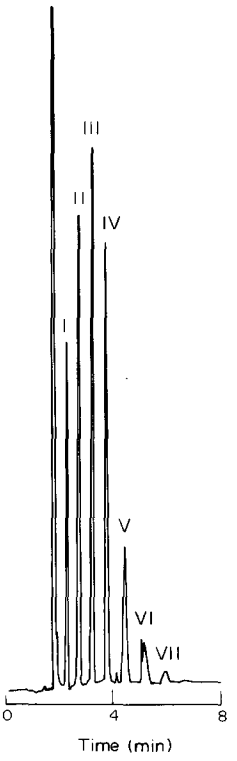

Figure 5
Electropherogram of 5.8 mM β-CD-SPE (4) introduced by pressure injection at 5 psi for 1 s. The field strength was maintained at 333 V cm^{-1}. The roman numerals above each peak indicate the degree of substitution the peak represents. The response was measured as a *loss* of absorbance.

stitution expected from molar ratios of the reactants, yet accounted for no more than 25% (w/w) of the total material. These results clearly demonstrate the usefulness of capillary electrophoresis for the characterization of these poly substituted β-CDs.

Conclusions

The capillary electrophoresis technique presented in this paper provides a method for the characterization of poly substituted sulphoalkyl ether β-CD derivatives. It allowed comparisons of each degree of substitution which composed the mixture and as such provided an excellent 'fingerprint' of the derivatized material. In principle, this technique could be used to separate any derivatized β-CD bearing a charge.

As migration of the sulphoalkyl ether of β-CD through the capillary is retarded by the anionic charge of the species, any compound which forms an inclusion complex with the derivatized β-CD should also be selectively retarded. Retention of non-ionic species not previously amenable to analysis by capillary electrophoresis should be possible from inclusion with the derivatized β-CD. Also, enhanced separation of enantiomeric compounds should result for compounds which form inclusion complexes with these derivatized β-CD. Studies have been completed in this laboratory, and will be presented in subsequent papers, which show that the sulpho butyl ether derivatives of β-CD will provide separation of racemic mixtures not previously possible with β-CD alone.

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