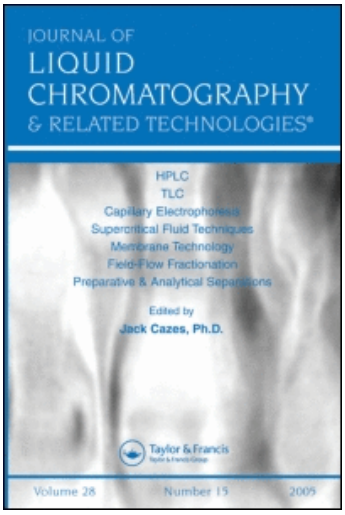

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DETERMINATION OF SODIUM ACETATE IN ANTISENSE OLIGONUCLEOTIDES BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

The determination of sodium acetate in antisense oligonucleotides purified by reverse phase chromatography in acetate buffer is important in both impurity profiling and drug concentration assay. A method has been developed for the quantitation of sodium acetate in a 20-mer phosphorothioate oligonucleotide drug substance employing capillary zone electrophoresis. Chloride was included as an internal standard to increase accuracy and precision of the measurement. Interference due to oligonucleotide matrix was eliminated by filtering the olignucleotide solution through a Centricon SR-3 column. The linear dynamic range for the method is 0.06-240 μg/mL. The electrophoretic separation requires only 6 min. The method has been demonstrated to be simple and reliable for the quantitation of acetate salts in oligonucleotide drug substances.

INTRODUCTION

Antisense oligonucleotides have been developed in recent years as potential drugs to treat human diseases ranging from HIV to cancer.[1] The specific binding of oligonucleotides to their corresponding RNA targets via Watson-Crick hybridization can inhibit RNA splicing[2] and mRNA translation,[3] and cause the degradation of RNA by RNase H.[4] Phosphorothioate oligonucleotides are one class of first-generation antisense analogs of natural oligonucleotides, modified to enhance nuclease resistance while maintaining hybridization to the target mRNA.[5] Phosphorothioate oligomers are generally synthesized on a solid phase DNA synthesizer and purified by preparative reverse phase chromatography. Sodium acetate is introduced by the elution buffer and removed by subsequent ultrafiltration and/or precipitation. The determination of residual acetate in the purified oligonucleotide drug substance is important for drug purity analysis.[6,7]

There is a lack of simple methodology for determination of acetate at low concentrations.[8,9] Various chromatographic techniques have been applied for the quantitative determination of acetate. However, they are less advantageous due to requirements for (i) extensive sample preparation or (ii) appropriate derivatization in order to introduce a detectable chromaphore or to reduce the analytical polarity.[10-13] A hyphenated GC-MS technique has been proposed for the determination of acetate in dried solid samples, in which the acetate was converted to acetic acid by heating with oxalic acid, followed by chromatographic purification, combustion and mass spectrometric detection of carbon dioxide. The recoveries are only 81 $\pm$ 5 % in the linear dynamic range of 3-50 μmoles of acetate.[14] Nonaqueous titration has been suggested by the United States Pharmacopeia for the determination of acetate salts, but the method is only suitable for distinctly anhydrous salts in the absence of other carboxylate anions,[15] not applicable to oligonucleotides. Membrane electrode sensors for the determination of acetate suffer from a narrow dynamic range, poor sensitivity and serious interferences.[16,17] A potentiometric gas sensor was proposed for the determination of acetate in medical solutions, metal acetates and vinegar. The device is composed of a gas-permeable teflon membrane and an internal pH sensing electrode cell. Acidifying the acetate solution to pH < 2 allows the free acetic acid vapor to diffuse through the membrane and changes the pH of the electrode.[8] ^{1}H NMR spectroscopy has been used for the determination of acetate in intravenous infusions and haemodialysis solutions, based on the singlet spectrum of the methyl protons of acetate in D_2O at δ 1.92 with N-methylurea as internal standard.[9] Unfortunately, the NMR method can only be used for solid samples at high acetate concentrations, and is not feasible for quantitating acetate in oligonucleotides due to interference by the oligonucleotide matrix. An enzymatic spectrophotometric method for direct

kinetic assay of acetate in serum or plasma is based on three sequential enzymatic reactions involving acetic kinase, pyruvate kinase and lactate dehydrogenase. The acetate concentration was measured by the decrease in absorbance at 340 nm due to NADH consumption.[18] The requirement of using three enzymes for the determination made this method less attractive.

Capillary electrophoresis is one of the fastest growing separation techniques today.[19,20] Analytes include simple organic and inorganic molecules, peptides, proteins, oligonucleotides, and DNA fragments.[21] One advantage of this technique is the high analytical efficiency, which is orders of magnitude greater than those obtained by HPLC and is achieved in a similar or shorter period of time.[21] Capillary Zone Electrophoresis (CZE), the simplest and most widely used CE mode, provides separation based on differences in free-solution mobility. In the present work, CZE has been developed as a simple and reliable method for the determination of residual acetate salts in phosphorothioate oligonucleotides.

MATERIALS AND METHODS

Deionized (Milli-Q) water was used for the entire experiment unless otherwise stated. The CIA-Pak™ Anion-BT solution was purchased from Waters Corporation (Milford, MA). Potassium hydrogen phthalate and sodium acetate trihydrate (HPLC grade) were purchased from Sigma (St. Louis, MO). Sodium chloride was purchased from J.T. Baker (Philipsburg, NJ). Centricon-SR3 (3,000 MW) tubes and 0.2 micron filters were purchased from Amicon, Inc. (Beverly, MA) and Nalge Company (Rochester, NY), respectively. The oligonucleotide sample is a 20-mer phosphorothioate of the sequence 5' GTT CTC GCT GGT GAG TTT CA 3'. It was synthesized using a Milligen 8800 DNA synthesizer.

(1) Preparation of Electrolyte and Reference Standard Solutions

Phthalate electrolyte solution was prepared weekly by weighing 0.204 g potassium hydrogen phthalate into a beaker and adding 5 mL of Waters CIA-Pak™ OFM Anion-BT solution and about 150 mL Milli-Q water. The solution was mixed thoroughly and the pH was adjusted to 5.6, using 1 N NaOH. The solution was transferred into a 200 mL volumetric flask, filled to the mark with Milli-Q water and filtered through a 0.2 micron filter. Acetate stock solution (2.00 mg/mL) was prepared by weighing 4.611 g sodium acetate ($CH_3COONa \cdot 3H_2O$, MW 136.08, HPLC grade) into a clean 1000 mL

volumetric flask, dissolved and filled to the mark with Milli-Q water. The stock solution was stored at 2-8°C when not in use and kept for a month. Chloride stock solution (4.00 mg/mL) was prepared by weighing 6.594 g sodium chloride into a clean 1000 mL volumetric flask, dissolved and filled to the mark with Milli-Q water. The solution was stored at 2-8°C when not in use and kept for 6 months. Reference standard solutions of acetate in the range of 0.06-240 μg/mL, containing 40 μg/mL chloride as internal standard, were prepared by transferring appropriate volumes of the acetate and chloride stock solution to a volumetric flask and diluting to the mark with Milli-Q water.

(2) Preparation of Sample Solutions

Solutions of oligonucleotide drug substance were prepared by accurately weighing 5 ± 0.1 mg of oligonucleotide sample. Concurrently, an additional sample was weighed for the determination of water content by capillary gas chromatography. About 3 mL of Milli-Q water were added into a 5 mL volumetric flask containing the oligonucleotide. The sample was dissolved and mixed well by vortexing. 50 μL of the 4.00 mg/mL chloride stock solution was added and the sample solution was diluted to the mark with deionized Milli-Q water and mixed well by shaking.

The sample solution was filtered through Centricon-SR3 3,000 MW tubes to remove the oligonucleotide matrix. Prior to fitration of oligonucleotide solution, the Centrion SR-3 tube was washed twice by centrifugation of 2 mL deionized Milli-Q water at 5,000 rpm for 60 min, and the residual water was removed by inverting the tube and centrifuging for 5 min at the same speed. About 2 mL of oligonucleotide solution was added to the clean Centricon tube and centrifuged at 5,000 rpm for 60 min at 4°C. The collected solution in the bottom of the tube was analyzed by capillary zone electrophoresis to determine acetate.

(3) Capillary Electrophoresis Apparatus

A polyimide coated fused silica capillary tube of 100 μm i.d. was cut to 60 cm length. A detection window was made by burning an approximate 5 mm section at 15 cm from one end using an electrical heating device to remove the coating. The column was rinsed for 10 min with 1 N NaOH and 10 min with Milli-Q water, filled with a 0.2% (v/v) solution of γ-methacryloxypropyltrimethoxysilane in 50% (v/v) ethanol/water, and allowed to stand for 1 hour.

The column was then flushed with air to remove residual reagent and rinsed with Milli-Q water for 10 min. The capillary column was placed in a capillary cartridge. Both ends were cut to form a 47 cm column (40 cm from inlet to the detection window).

A Beckman P/ACE 5000 instrument (Fullerton, CA) was used for the CZE experiments. As the capillary cartridge was inserted, the column was rinsed with Milli-Q water for 2 min before use. Under high pressure, the capillary column was filled with electrolyte solution for 30 seconds before each sample injection and was rinsed with Milli-Q water for 30 seconds after each run. The sample solution was injected hydrostatically for 10 seconds and separated at 15 kV for 6 min at 30°C. The background absorbance was measured at 254 nm.

RESULTS AND DISCUSSION

Phthalate is an intermediate mobility electrolyte which absorbs UV light at 254 nm. CIA-Pak™ Anion-BT is an electroosmotic flow modifier used to reverse the normally cathodic direction of the electroosmotic flow found in fused-silica capillaries.[22] Phthalate (5 mM) containing 2.5% (v/v) CIA-Pak™ OFM Anion-BT was applied as cathode and anode electrolytes and filled into the capillary tube. As the sample solution was injected hydrostatically and the electric field was applied, all anions migrated toward the anode. Since the capillary tube contained the same concentration of phthalate, a steady absorbance of phthalate was obtained. Acetate in the sample plug migrates faster than phthalate. Since the anions repel each other, phthalate ions were replaced by the acetate ions during the migration process. Acetate exhibits no absorption of UV light, therefore the relative decrease in phthalate concentration is attributed to a decrease in absorbance as acetate ions reach the detector.

Using negative detector polarity, a peak is obtained whereby the peak area is proportional to the acetate concentration in the sample. Since the oligonucleotide samples do not contain chloride, chloride has been selected as an internal standard and added to the sample solution. The chloride ions migrate faster than acetate ions (Figure 1). The area of acetate has been normalized against the area of chloride, the internal standard and the normalized area of acetate has been used for quantitation. The relative migration time of acetate to that of chloride (the normalized migration time) is used as an identity for acetate.

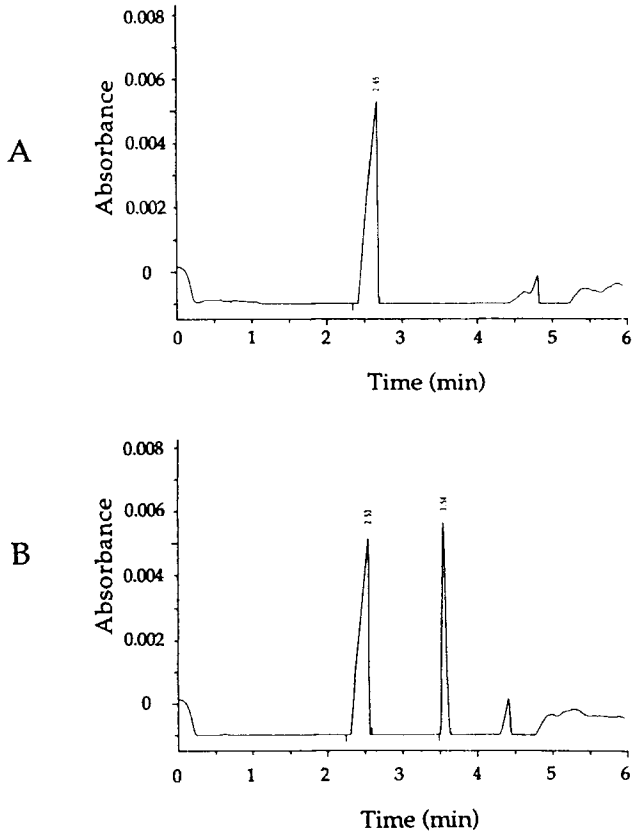

Figure 1. Electropherograms of a reference standard solution containing 40 μg/mL chloride as internal standard and (A) 0 μg/mL acetate, (B) 20 μg/mL acetate.

Oligonucleotides are negatively charged and migrate under the electric field. They have an absorption at 254 nm, and hence interfere with the determination of acetate in two ways: positive response due to replacement of phthalate ions and negative response due to absorption at high concentrations. Figure 2 depicts the electropherogram of a 1 mg/mL oligonucleotide sample solution where the peaks of acetate and added chloride in the sample solution were fully covered. The strong interference of the oligonucleotide with acetate was removed by prior centrifugation of the sample solution through a Centricon-SR3 (3,000 MW) filter. As the molecular weight of the oligonucleotide is significantly larger than 3,000 Da, the oligonucleotide cannot pass through the filter and is separated from acetate ions.

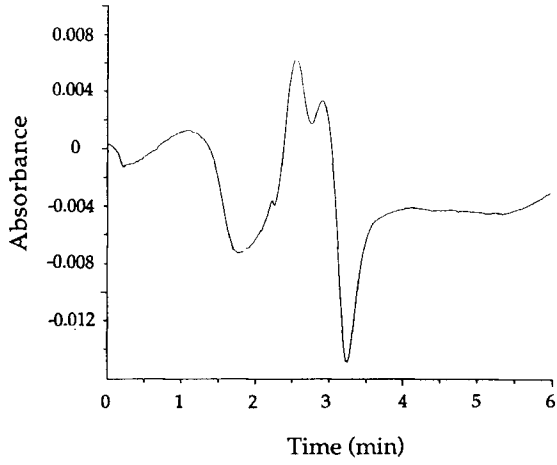

Figure 2. Electropherogram showing the interference of ISIS 3521 oligonucleotide matrix.

Before use, the Centricon SR-3 column was cleaned by washing twice with deionized Milli-Q water. After centrifugation, the collected solution in the bottom of the tube was used for the analysis by capillary zone electrophoresis. Figure 3 demonstrates the effectiveness of the Centricon SR-3 filter for the isolation of acetate and chloride ions from the oligonucleotide matrix without any contamination of the sample solution. No signal was obtained as Milli-Q water was eluted from the column (Figure 3-A). An electropherogram of the sample solution obtained following passage through the Centricon SR-3 filter (Figure 3-B) indicates that the interference by oligonucleotide is fully eliminated and chloride and acetate peaks obtained are similar to those of an acetate standard solution as depicted in Figure 1-B.

The purpose of adding chloride as an internal standard is to identify the acetate by relative migration time and to improve precision. Table 1 shows the migration time of acetate and chloride. The migration time of acetate varied during the measurements (3.217-3.487) while the relative migration time, which is defined as the ratio of the migration time of acetate to that of chloride, was approximately 1.38. The relative standard deviations (n=6) were 2.95 % for migration time of acetate and 0.66 % for the normalized migration time, respectively, indicating that migration time relative to chloride can be used to effectively identify the position of the acetate peak in the electropherogram.

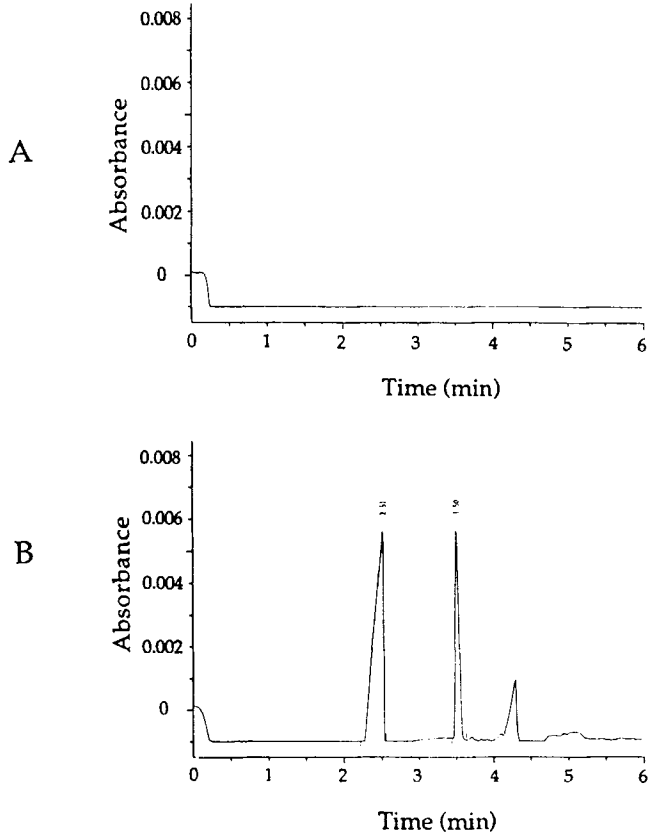

Figure 3. Electropherograms of (A) a deionized water blank and (B) an ISIS 3521 (Lot #0027) sample solution following passage through the Centricon SR-3 column.

The relative percent difference of the normalized migration time between the sample and the reference standard was less than 2%. Table 2 lists the results obtained for five repetitive injections of the same sample. The relative standard deviations were 4.39% for the acetate peak area and 1.88% for the normalized peak area. The precision of the method was improved considerably by using normalized peak area for quantitation.

Acetate samples were injected in hydrostatic mode. The injection time was tested in the range of 0-20 seconds and a linear relationship between the peak area and the sample injection time was found between 0-15 seconds. A sample injection time of 10 seconds was chosen as a compromise between

Table 1

The Migration Time of Acetate and Chloride in the Sample Solution

Lot Number	Migration Time (Min) Chloride	Acetate	Normalized Migration Time
	2.403	3.317	1.380
0027	2.453	3.427	1.397
	2.513	3.487	1.388
	2.333	3.217	1.379
0028	2.353	3.297	1.401
	2.463	3.407	1.383
RSD (%):	2.86	2.95	0.66

Table 2

Reproducibility for Repetitive Injections (n = 5)

	Acetate	Chloride	Normalized (Acetate/Chloride)
	0.2855	1.123	0.2542
Peak	0.2598	1.030	0.2522
Area	0.2808	1.104	0.2542
	0.2883	1.186	0.2432
	0.2901	1.142	0.2539
RSD (%):	4.39	5.12	1.88

sensitivity and resolution. Voltage was evaluated in the range of 0-20 kV. The higher the voltage, the shorter the separation time, but high noise was observed as the voltage was increased to 20 kV. Therefore, a voltage of 15 kV was selected for the separation. Under these conditions, the retention time for acetate is about 3.5 min. A calibration curve is obtained by plotting normalized area of acetate versus the concentration of acetate standard solutions in μg/mL. Linearity was demonstrated in the range of 0.06 to 240 μg/mL acetate with a y-intercept of -0.252, slope of 0.0624 and a correlation coefficient of 0.994. The recovery experiment was studied by the quantitation of Centricon-SR3 column

filtrates having known concentrations of acetate and by the standard addition method. The recovery is above 96%. Detection limit, estimated from the lowest concentration standard (0.06 μg/mL), is approximately 0.0012% w/w acetate, equivalent to 0.0017% w/w sodium acetate. Three typical oligonucleotide samples analyzed showed sodium acetate concentration of 1.43%, 2.60% and 0.058%, respectively.

CONCLUSION

Capillary Zone Electrophoresis has been demonstrated to be a simple, sensitive and reliable method to detect and quantitate the acetate salt present in oligonucleotide drug substances. Chloride was included as an internal standard for the identification and quantitation of acetate. As a result, both accuracy and precision have been increased as shown in Table 1 and 2. Although a single 20-base phosphorothioate oligonucleotide is shown as an example, the method can be applied to the determination of acetate in other oligonucleotides having molecular weights above 3,000 Da. Smaller molecular weight cut-off membranes are required to filter oligonucleotides with molecular weights less than 3,000 Da.

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