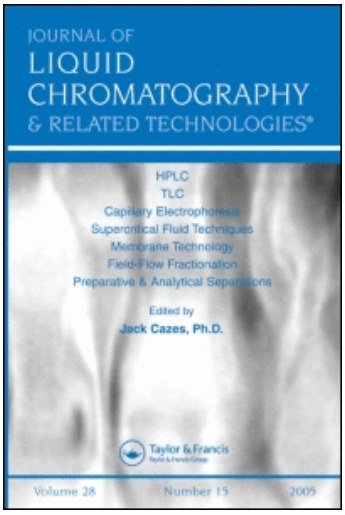

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Deoxyribonucleotides Using Ion Suppression High Performance Liquid Chromatography with a Reversed-Phase Column

R. P. Whitehouse[a]; C. L. Greenstock[a]

[a] Medical Biophysics Branch Whiteshell Nuclear Research Establishment Atomic Energy of Canada Research Company Pinawa, Manitoba, Canada

To cite this Article Whitehouse, R. P. and Greenstock, C. L.(1982) 'Separation of Deoxyribonucleotides Using Ion Suppression High Performance Liquid Chromatography with a Reversed-Phase Column', Journal of Liquid Chromatography & Related Technologies, 5: 11, 2085 — 2093

To link to this Article: DOI: 10.1080/01483918208067619

URL: http://dx.doi.org/10.1080/01483918208067619

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2085-2093 (1982)

SEPARATION OF DEOXYRIBONUCLEOTIDES USING ION SUPPRESSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A REVERSED-PHASE COLUMN

R.P. Whitehouse and C.L. Greenstock

Medical Biophysics Branch
Whiteshell Nuclear Research Establishment
Atomic Energy of Canada Research Company
Pinawa, Manitoba, Canada ROE 1LO

ABSTRACT

Ion suppression-reversed phase high performance liquid chromatography, using 0.6 M ammonium dihydrogen phosphate as eluent, produces base-line separations of deoxyribonucleotides. The effects of pH and ionic strength are described. This isochratic system is simple, reproducible and fast, requiring less than 30 min for a complete separation, and is suitable for *in vitro* studies.

INTRODUCTION

Conventionally, nucleotides have been separated by ion exchange on anion columns. Hori(1) and Murakani (2) demonstrated anion exchange chromatography, with totally porous packing in the late 1960's, which usually required many hours for separation. Ion exchange chromatography is based on a difference in affinity of the solute ions for the stationary phase, which depends upon the relative rate

of distribution of the ionic solute between the mobile phase and the stationary phase. Recently, Brown, Hartwick and Krstulovic (3), using this principle, have separated and analyzed nucleotides from whole blood using ion exchange chromatography. Normally, reversed-phase columns have been used for separating non-ionic compounds on bonded non-polar stationary phases (4) and were first used in describing liquid-liquid partition separation of fatty acids on paraffin oil and n-octane using aqueous eluents (5). However, with recent advances in HPLC, utilizing reversed-phase columns showing their versatility, simplicity and reproducibility, samples containing ionic species have been separated by the use of ion suppression or ion pair chromatography (IPC) (6). Ion suppression is used in the separation of weakly acidic and basic compounds by driving the equilibrium to the non-ionic side by adjusting pH with buffer. Under such conditions, therefore, a reversed-phase column can be used to separate ions as well as non-ionic compounds. This note describes the application of ion suppression to reversed-phase HPLC for the isocratic separation of six deoxyribonucleotides: deoxycytidine 5'-monophosphoric acid (d-CMP), deoxyuridine 5'-monophosphoric acid (d-UMP), deoxyguanosine 5'-monophosphoric acid (d-GMP), thymidine 5'-monophosphoric acid (d-TMP), 5-bromodeoxyuridine 5'-monophosphoric acid (d-BrUMP), and deoxyadenosine 5'-monophosphoric acid (d-AMP).

Adequate base-line separation was only achieved by using very high salt concentrations (0.6 M) under conditions not previously reported in the literature. Practical

details are given of the conditionings, operations and regeneration of reverse-phase columns used under conditions of high ionic strength.

MATERIALS AND METHODS

Chemicals

The 5'-deoxyribonucleotides (sodium salts) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Ammonium dihydrogen phosphate (monobasic), certified A.C.S. was from Fisher Scientific Company Ltd (Winnipeg, Canada). All solutions were made up in distilled water.

Apparatus

The reversed-phase column used in these studies was a Li Chrosorb RP-8, (250 mm x 4.6 mm) from Unimetrics Corp. (Anaheim, CA, USA). The variable UV wavelength detector used was a model 837 (Dupont Co., Wilmington, DE, USA) set to monitor absorbance at 265 nm. A Rheodyne sample injector valve with a fixed 20 µL sample loop, model 70 - 10, was from Applied Science Lab (State College, PA, USA). The pump used was a model 3100 (Chromatronix Inc., Berkely, CA, USA) pressure vessel pump which supplies the liquid by applying gas pressure, usually nitrogen, to the surface of the mobile phase. The chromatograms were recorded on a model SR-255B Heath recorder at a chart speed of 0.1"/min at an output voltage of 10 mV.

Chromatographic Conditions

The reversed-phase (Li Chrosorb RP-8) column was conditioned by washing with distilled water for 15 min, then 15 min with 100% methanol followed by a further washing with distilled water for 15 min. The flow rate during the column preparation was 0.5 mL/min at a pressure of 6200 KPa at ambient temperature. The eluting solvent 0.6 M $NH_4H_2PO_4$ (pH 4.25) was run through the column for 30 min to allow the column to stabilize. A 20 μL sample of a mixture of $\approx 1.7 \times 10^{-4}$M of each deoxyribonucleotide (≈13 μg), was injected onto the column using a Rheodyne sample injector valve, and could be readily detected at an attenuation of 0.32 absorbance units (AU), on the detector.

At the end of each day, the column was washed with distilled water for 15 min, to remove the high salt concentration. This washing is necessary to prevent the silica backbone of the packing material from being damaged by the high concentration of the phosphate ions, in an analogous manner to a basic eluent above pH 7.5 (7). With constant running of samples, over a period of a week, the retention times of the individual deoxyribonucleotides decrease and resolution between d-GMP and d-TMP is lost. In order to avoid this, the column must be regenerated. This is accomplished with a 15 min distilled water wash followed by a 100% methanol wash, and finally another 15 min distilled water wash. The flow-rate during the regeneration is the same (0.5 mL/min) as the analysis.

RESULTS AND DISCUSSION

The final operating conditions were arrived at by optimizing the salt concentration over a range of from 0.05 - 0.8 M at pH 3.5. The higher the ionic strength, the greater the retention time for the solutes. The 0.6 M salt concentration gave the best separation at pH 3.5. The pH was then varied from pH 2 - 5 at a salt concentration of 0.6 M, and pH 4.25 was found to be optimum. To our knowledge, this salt concentration is higher than that used previously to separate nucleic acid derivatives(8). In our experiments using 0.4 M ammonium dihydrogen phosphate at pH 3.5 as described by Wakizaka (8), d-GMP and d-TMP would not separate. Increasing the ionic strength and pH of the ammonium dihydrogen phosphate decreased the retention times of the deoxyribonucleotides thus giving us adequate separation as demonstrated in Fig. 1. With careful conditioning, the retention times were reproducible to $\pm$ 1 min for the deoxyribonucleotide standards, over a weekly period before regeneration was needed.

Figure 1 illustrates the baseline separation of the deoxyribonucleotides eluting in the following order, with retention times shown in brackets: d-CMP (9 min), d-UMP (11.5 min), d-GMP (17 min), d-TMP (19 min), d-BrUMP (23 min) and d-AMP (28 min).

A series of dilutions of the 10^{-3} M stock solution of the deoxyribonucleotides were prepared and 20 µL aliquots of each were chromatographed under the conditions described in the Materials and Methods section, to generate calibration curves for each deoxyribonucleotide.

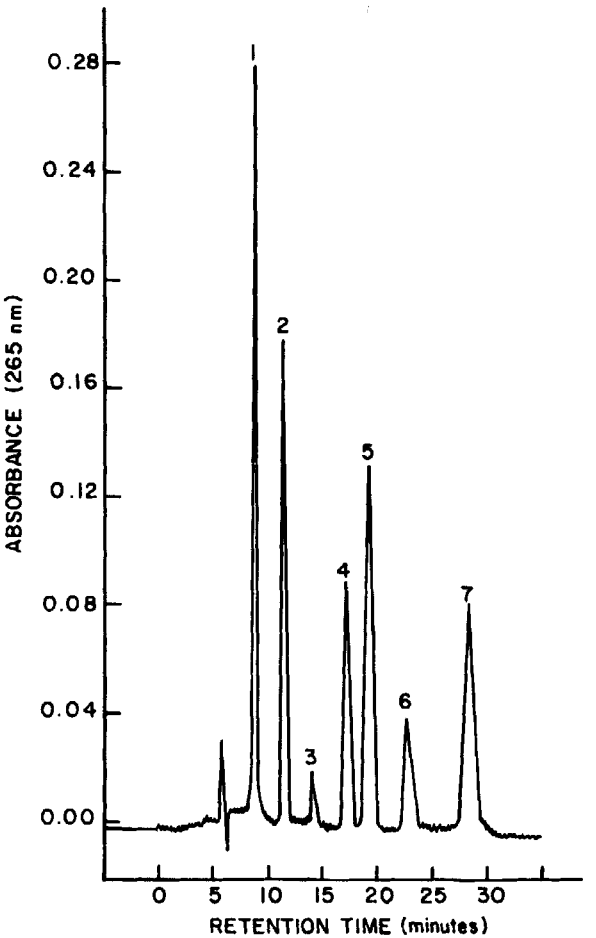

FIGURE 1. Separation of six standard deoxyribonucleotides: 1. d-CMP, 2. d-UMP, 3. Impurity, 4. d-GMP, 5. d-TMP, 6. d-BrUMP, 7. d-AMP. Column, 250 mm x 4.6 mm I.D., Unimetrics RP-8, 7 µm; eluent, 0.6 M $NH_4H_2PO_4$ (pH 4.25); flow-rate, 0.5 mL/min; pressure, 6200 KPa; temperature, ambient; sample volume, 20 µL; detection 265 nm.

Approximately 1.5 to 20 μg of the deoxyribonucleotide was injected and run at an attenuation of 0.16 AU. For cell studies, samples of ≈ 10^6 cells/mL can yield sufficient amounts of deoxyribonucleotides, following extraction and hydrolysis, to provide satisfactory results at an equivalent sensitivity.

UV spectra of the stock solutions were obtained using a Cary 15 spectrophotometer, and the actual concentrations were determined from published extinction coefficients[9] using Beer's law. The stock solutions were diluted 20 times to obtain a spectrum. The tabulated results are shown in Table 1. From Table 1, it is shown that the actual concentrations may vary from the theoretical concentrations calculated on the basis of molecular weights. The calibration curves serve to determine correction factors necessary for obtaining the true deoxyribonucleotide concentrations. In addition, the calibration curves also demonstrate the high degree of linearity and reproducibility of this HPLC system. The results are obtained using a high salt concentration in the solvent, and this is an advantage for physiological or biological samples, which can be injected with no adverse salt effects, directly onto the column without the necessity of dialysis.

In conclusion, the six deoxyribonucleotides have been successfully separated by a simple, reproducible system of isocratic reversed-phase chromatography employing ion suppression, and the total time for separation is less than 30 min.

TABLE 1. DETERMINATION OF CORRECTION FACTORS USED TO MEASURE CONCENTRATIONS OF STOCK SOLUTIONS OF THE DEOXYRIBONUCLEOTIDES

Deoxy-Ribonucleotides	O.D. 20X Diluted pH 7.0	Final O.D.	λ max (nm)	Extinction Coefficient	Actual conc. mg/10 mL	Calculated conc. mg/10 mL	Correction Factors
d-CMP	0.482	9.64	268	9,300	4.94	4.77	1.04
d-UMP	0.489	9.78	260	9,800	3.96	3.97	1.00
d-GMP	0.565	11.30	250	13,300	3.33	3.92	0.85
d-TMP	0.613	12.26	265	10,200	4.30	3.58	1.20
d-BrUMP	0.294	5.88	275	9,100	3.05	4.72	0.65
d-AMP	0.793	15.86	258	15,300	4.54	4.38	1.04

There is a continuing need for a sensitive method to resolve nucleotide derivatives in DNA extracts for a variety of studies in molecular biology and radiobiology. This method is being used to study enzymatically hydrolyzed DNA's in normal and transformed cells. Also, this assay is being used to measure the incorporation of d-BrUMP into the DNA of mammalian cells, and to test its effects on cellular radiosensitivity and transformation frequency.

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