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Separation of dideoxyribonucleosides in trace amounts by automated liquid chromatography and capillary electrophoresis

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ABSTRACT

No satisfactory high-performance liquid chromatographic (HPLC) method is currently available for the separation of the major dideoxyribonucleosides (ddNs) and their derivatives. A method involving HPLC has been developed for the separation of five major ddNs [ddA, ddC, ddI, azT and 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T)]. Elution of the common and modified components of DNA was also examined under the selected separation conditions of HPLC. The elution characteristics of these compounds were studied using serum plasma samples spiked with ddN derivatives. In addition, capillary electrophoresis (CE) was investigated for the separation of ddNs and their derivatives. Picomolar amounts of the five major ddNs and the metabolic product of azT [5′-O-glucuronide-3′-azido-3′-deoxythymidine (Glo-azT)] were satisfactorily resolved in 10 min by using a modification of CE. The spectral properties of the ddNs were characterized under different pH conditions and compared with those of their parent deoxyribonucleosides (dNs) because these compounds are commonly measured in HPLC by their spectral properties. The spectra of ddC and ddT derivatives resemble very closely those of dC and dT, but those of ddA and ddI differ to some extent from their parent dNs. The HPLC method was extensively examined for satisfactory resolutions of these compounds. For example, an isocratic elution method, although simple, failed to resolve these compounds and ion-pair chromatography did not offer any advantage. Gradient elution involving buffered solutions and increasing amounts of an organic modifier yielded satisfactory results. Methanol appeared to be the organic modifier of choice. A reversed-phase matrix with smaller than octadecyl alkyl chains did not produce the necessary interactions. Uniform spherical beads of smaller diameter produced superior resolutions. The separation of these compounds on three commercially available columns is discussed. The separation of human plasma samples spiked with dideoxynucleoside derivatives by HPLC was accomplished in *ca.* 16 min. The presence of the dNs did not interfere in their separations.

INTRODUCTION

Simple, rapid, and accurate methods for the separation of dideoxyribonucleoside derivatives are needed to study the compounds directed towards suppressing human immunodeficiency virus (HIV). These methods are necessary to provide pharmacokinetics in patients with AIDS and related disorders. The distribution of 3′-azido-3′-deoxythymidine (azT) and its metabolic end product, 5′-O-glucuronide-3′-azido-3′-deoxythymidine (Glo-azT) in blood plasma and urine samples and different organs has been examined by numerous investigators using high-performance liquid chromatographic (HPLC) methods [1–13]. Pharmacokinetics of dideoxyribonucleoside derivatives [such as 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyuridine (ddU) and 2′,3′-dideoxyinosine (ddI)] have also been studied by using different HPLC methods [14–20]. Other techniques used for the study of these compounds include radioactive tracers [21], enzyme-linked time-released fluoroimmunoassay [22,23], and liquid chromatographic–thermospray mass spectrometric methods [24,38].

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In addition, 3′-azido-2′,3′-dideoxy-5-methylcytidine [25] and 3′-azido-2′,3′-dideoxythymidine [26] have also been separated by HPLC methods. A two-column HPLC method was recently employed for the separation of mono-, di- and triphosphates of azT [27].

The separation method of choice for the dideoxyribonucleosides (ddNs) appears to be HPLC and it has been used more extensively than other methods. Recently, Jang and Brown [28] described guidelines for predicting the resolutions of different ddNs and 3′-azido-3′-deoxy thymidine monophosphate (azTMP) in HPLC separations. In addition, they examined the effects of temperature, eluent pH and methanol. At present, no satisfactory HPLC method is available for the separation of major ddN derivatives. Here, we describe the development of an HPLC method for the separation of five drugs of major clinical significance [ddA, ddC, ddI, azT, and 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T)] and demonstrate the elution of the common and modified components of DNA under the selected separation conditions.

Electrokinetic chromatography is applied to both HPLC and capillary electrophoresis (CE). It is most effective for the separation of electrically neutral substances. Micellar electrokinetic capillary electrophoresis (MEKC) is most commonly used, and involves the electrophoretic migration of ionic micelles with a velocity different from that of the bulk solution [29–32]. In micellar EKC, the separation is based on differential partition of the solute between the micelles and the surrounding aqueous (polar) phase. Micelles display electrophoretic migration, often towards the sample electrode; the mobile polar solution (buffer) on the other hand, exhibits stronger movement towards the detector electrode because of electroosmosis. MEKC offers extremely high efficiency of separation in comparison with HPLC. In contrast, HPLC separation is based on the partition of the solute between the mobile polar phase and the stationary phase (non-polar in the reversed-phase mode). Not only does MEKC offers powerful separations of neutral substances, but it also enhances the selectivity of the ionic substances, in contrast to conventional CE. In this work, we examined the separation of the five representative ddNs and their derivatives by employing the MEKC principle. The separation of these compounds by MEKC is compared with that obtained by HPLC.

EXPERIMENTAL

Analytical system and data-processing equipment

The chromatographic system utilized a manual injection valve (Rheodyne Model 7125), a programmable mobile phase gradient-elution pump (Perkin-Elmer Series LC 4), a reversed-phase liquid chromatographic (RP-LC) column and a diode-array detector (Pharmacia LKB Model 2140 rapid spectral detector). A microcomputer (Zenith Model Z-248, IBM-AT compatible) was used to operate the detection unit and also to collect and store the absorption spectra of the effluent every 1.0 s with the aid of Pharmacia LKB Wavescan-EG software. Analyses were carried out using the diode-array detector. Here the results are shown as topograms, and for clarity some chromatographic peaks are shown at only two wavelengths (260 and 280 nm). Details of this automated equipment have been published elsewhere [33,34].

Chromatographic columns

Silica-based RP-LC (octadecylsilane, C_{18}) columns from three manufacturers were employed: Econosphere C_{18}, 5-μm beads (25 × 0.46 cm I.D.), from Alltech (Deerfield, IL, USA), Supelcosil C_{18}, 5-μm beads (25 × 0.46 cm I.D.), from Supelco (Bellefonte, PA, USA), and Vydac C_{18}, 5-μm beads (25 × 0.46 cm I.D.) and Vydac C_{18} (10-μm beads) (25 cm × 4.63 mm I.D.), from Separation Group (Hesperia, CA, USA). Each column was installed with a guard column.

Nucleoside samples

ddC, azT and Glo-azT were obtained from Sigma (St. Louis, MO, USA) and ddI, ddA and d4T were gifts from Dr. C. Sapino, Site Director, Bristol-Myers (Syracuse, NY, USA). Human plasma samples were obtained from normal donors (see below for sample preparation).

Preparation of dideoxyribonucleoside solutions

Concentrated stock solutions of ddNs were prepared in 1.0 ml of 20 m*M* succinate buffer (pH 5.5) in a 1.5-ml polyethylene micro centrifuge tube. To enhance the solubility of the purine derivatives and

that of d4T, 0.1 ml of methanol was added to the solution. Each solution was filtered through a 0.2-μm filter tip–syringe assembly (Millipore, Bedford, MA, USA). Necessary dilutions were carried out with appropriate solutions (0.1 *M* HCl or 0.1 *M* NaOH or a buffer of pH 5.5 or 7.0) and the UV spectrum of each sample under different pH conditions was measured. The spectra were collected from 200 to 310 nm with a wavelength step of 0.5 nm. For spectral analyses, a Gilford Response spectrophotometer (Ciba Corning, Oberlin, OH, USA) was used and the data were downloaded using Responstar software (Gilford-Corning) to an IBM-compatible microcomputer and the results were analyzed using a spreadsheet software Excel version 3.0a (Microsoft, Redmond, WA, USA). For HPLC analyses, artificial mixtures of known concentrations of the five ddNs and also six dNs were prepared by mixing appropriate amounts of each compound.

Preparation of normal human plasma samples

A human blood sample (1 ml), drawn using a tuberculin syringe and transferred to a tube containing EDTA, was centrifuged at 1000 *g* at 10°C for 10 min. The supernatant (plasma) was transferred into Eppendorf tubes using a micropipette and diluted (1:10) using physiological saline solution.

Separation of dideoxyribonucleosides and spiked plasma samples

The dideoxyribonucleoside mixture was analysed by HPLC utilizing solvents of different polarities [methanol, tetrahydrofuran (THF) and acetonitrile], different buffer solutions containing an ion-pairing reagent [octanesulphonic acid (OSA)], and different RP-LC columns (see above). Analyses were carried out by using different solvent systems (see Table II in Results). The eluent which produced the best separations contained 20 m*M* sodium succinate–succinic acid and 1 m*M* sodium azide (pH 5.50) and various amounts of methanol. A 5–10 μl-sample was applied by using a 25-μl sample loop.

Capillary zone electrophoresis system

The CE equipment, designed in this laboratory [35], consisted of the following four basic components: (a) a polyimide-coated fused-silica capillary tube (80 cm × 75 μm I.D. × 280 μm O.D.; 52 cm from sample-end electrode to the detector); (b) a specifically designed on-line Z-shaped flow cell with a light path of 3 mm (LC Packing, San Francisco, CA, USA); (c) a Spectroflow 757 variable-wavelength UV monitor (Kratos, Ramsey, NJ, USA), which was modified to accommodate the above-mentioned flow cell; and (d) a high-voltage power supply (0–30 kV) with features of programmable, reversible-polarity output (Bertan, Hicksville, NY, USA). The electric current was monitored by a digital multimeter (John Fluke, Everett, WA, USA). An elution pump (Waters Assoc., Milford, MA, USA) was used for washing the column and initiating the sample injection. The signals from the detector were fed to a strip-chart recorder for instant monitoring of the results. In addition, data were acquired with an IBM-compatible PC by using an analog-to-digital interface and retrieved with the help of software (Nelson Analytical, Cupertino, CA, USA).

Capillary zone electrophoresis operation

The capillary column was first washed by pumping a buffer through it and allowing the pressure to reach a constant value. The liquid flow was then disconnected and the capillary dipped into the sample contained in a miniature conical polyethylene tube. The positive liquid flow current generated by the pump and the gravitational force were enough to allow introduction of the sample into the fine capillary column. The sample flow was allowed for a specific time (between 5 and 20 s). This method of sample application was found to be very reproducible. For example, a 5-s injection introduced 3.0 nl of the sample into the capillary column. This value, having a standard deviation of 0.33 nl or 11%, was based on three different injections. The direction of the solution flow during the electrophoresis was selected by simply changing the polarity of the current.

RESULTS

Spectral properties of dideoxyribonucleosides

The absorption spectra of ddA, ddC, azT, d4T, and ddI under different pH conditions are shown in Fig. 1–5. The spectral properties of these compounds are compared with those of their parent

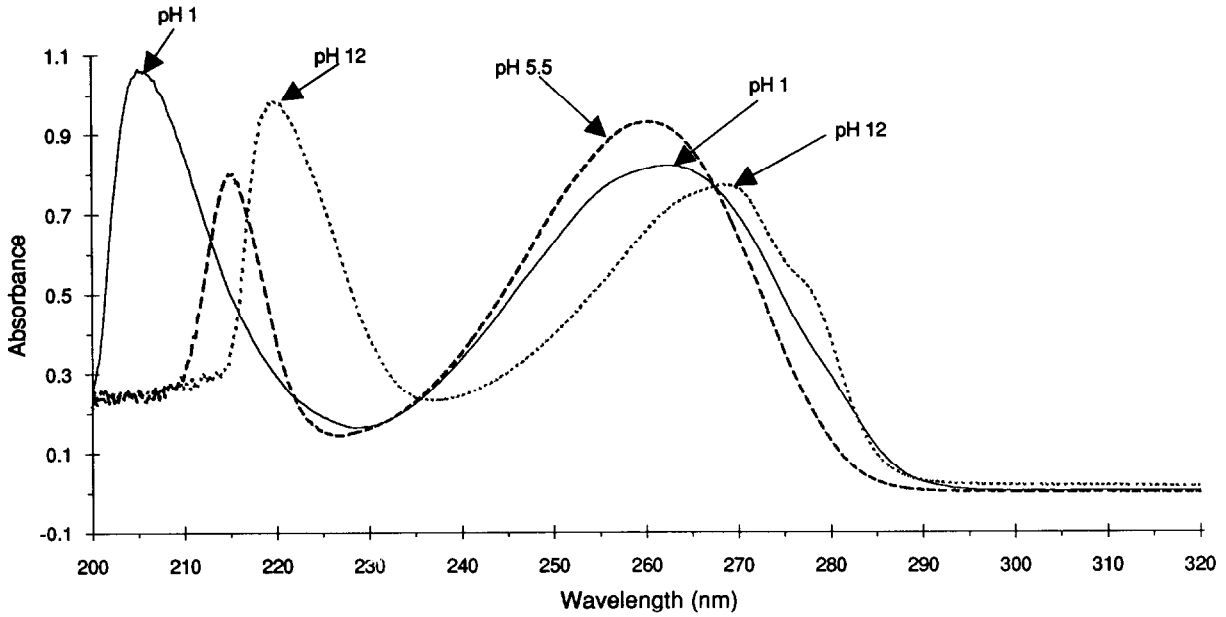

Fig. 1. Spectra of 2′,3′-dideoxyadenosine (ddA) at different pH values.

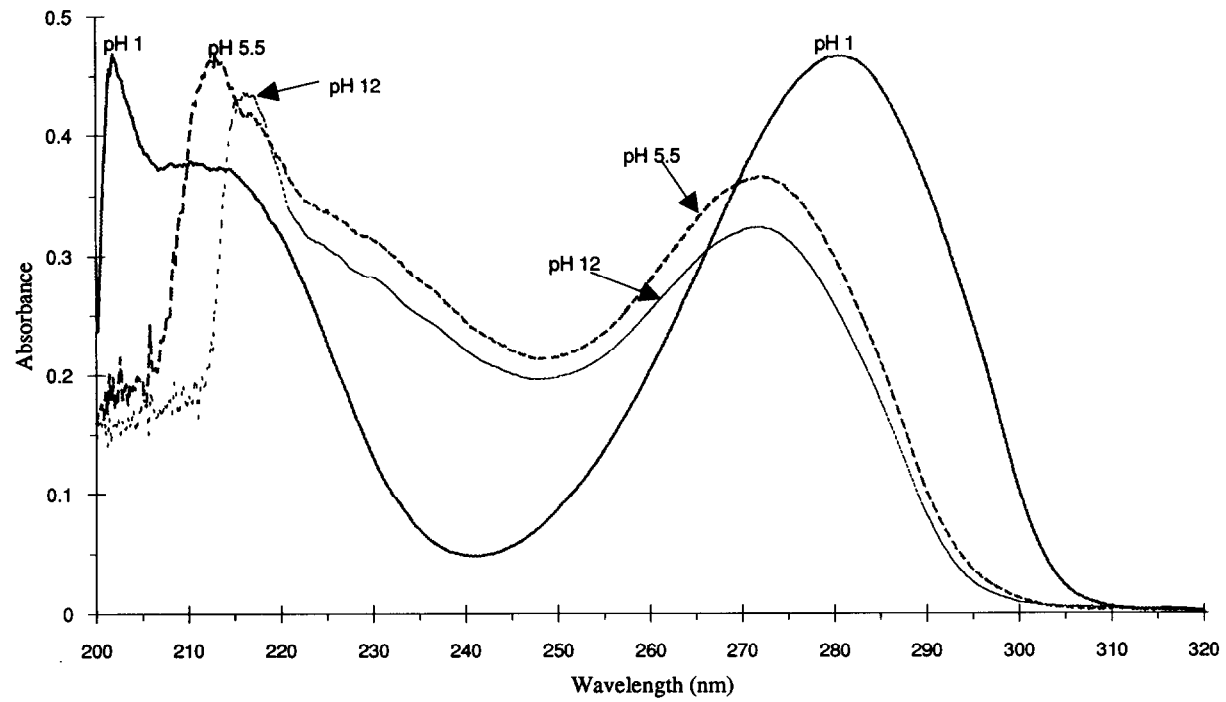

Fig. 2. Spectra of 2′,3′-dideoxycytosine (ddC) at different pH values.

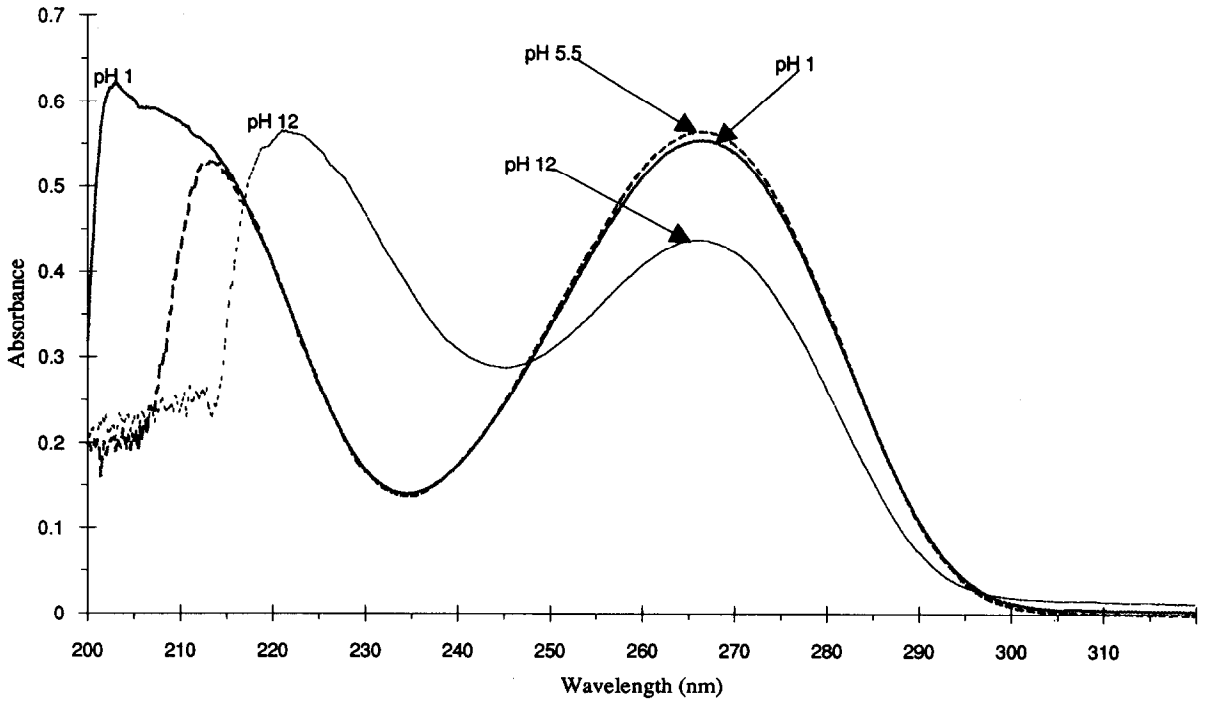

Fig. 3. Spectra of 3′-azido-3′-deoxythymidine (azT) at different pH values.

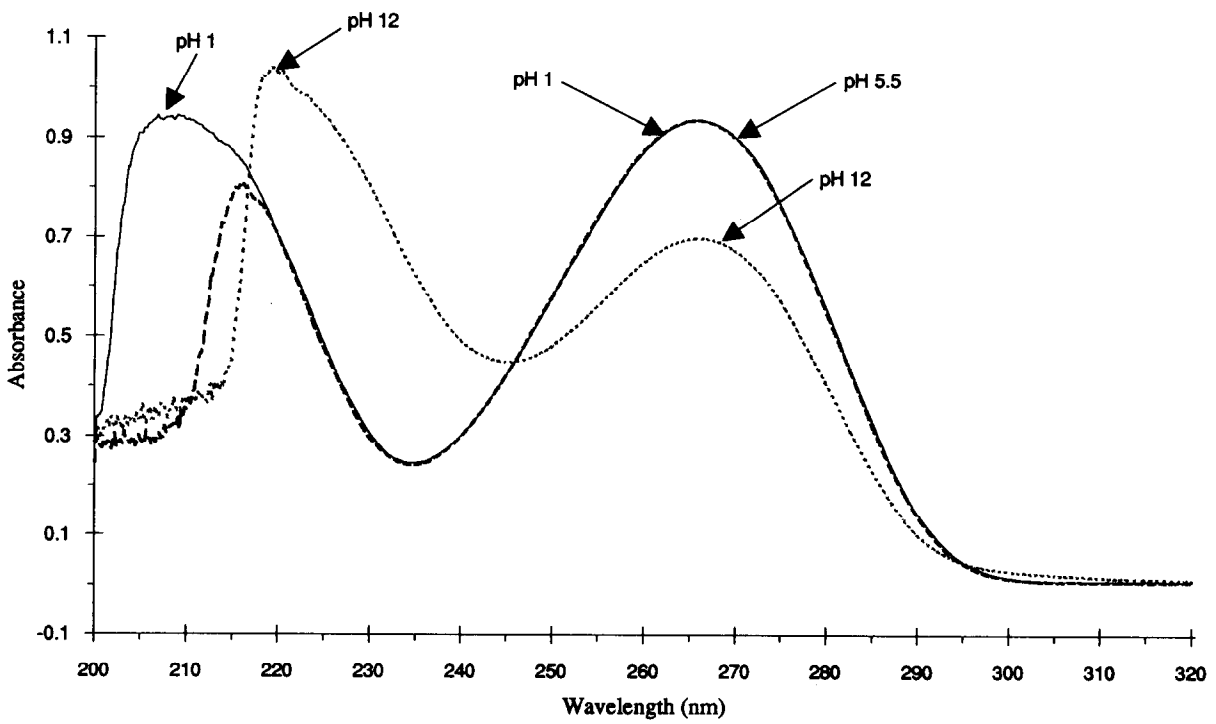

Fig. 4. Spectra of 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) at different pH values.

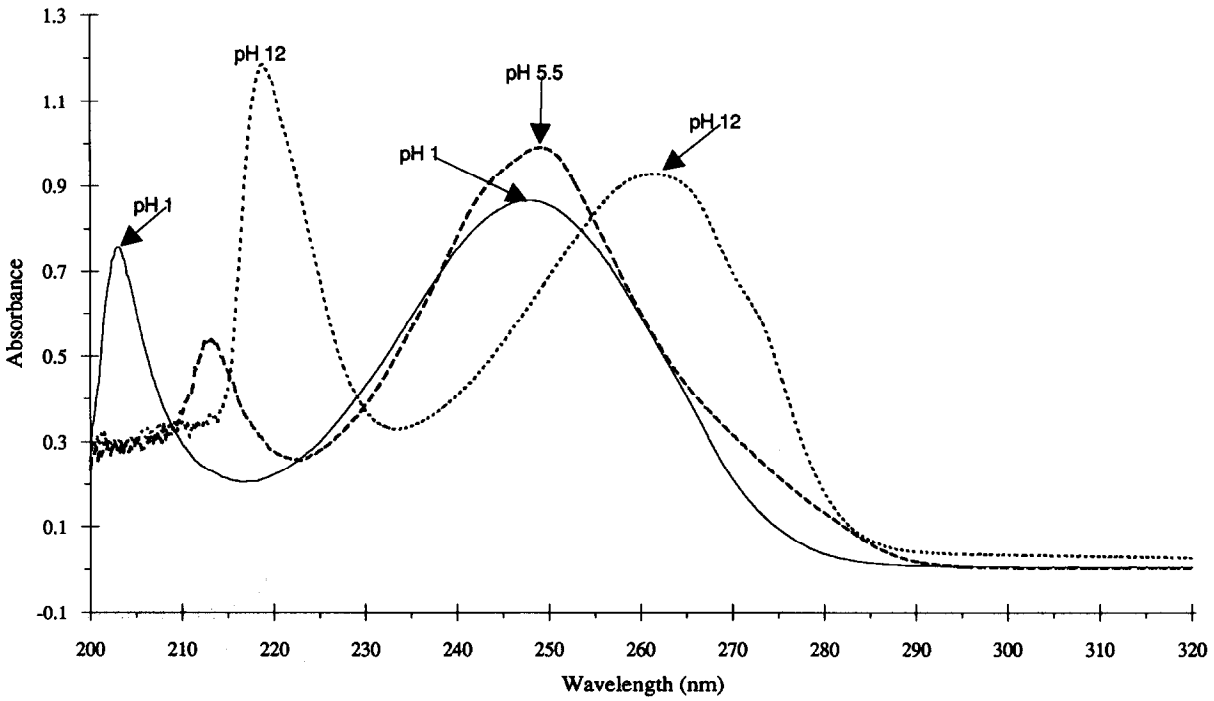

Fig. 5. Spectra of 2',3'-dideoxyinosine (ddI) at different pH values.

dNs in Table I. The ddA spectrum differs considerably from that of the parent compound, dA. Whereas the λ_{max} of ddA is shifted to a higher wavelength at pH 1 and markedly at pH 12, the λ_{max} at the neutral pH is changed to a lower wavelength (10 nm). The λ_{min} values under acidic and neutral conditions do not change between the two adenosine compounds, but the values differ under basic conditions. Although the ddI spectra are very similar to those of dI under acidic and neutral conditions, the λ_{max} of ddI shifts to a markedly higher wavelength under alkaline condition. The spectra of ddC resemble very closely those of dC and similarly the spectra of azT and d4T correspond to dT.

Development of HPLC methods

Effect of organic solvents in the mobile phase. The effect of three organic solvents having different polarities in the mobile phase was examined for the separation of a complex mixture of dNs and ddNs on a C_{18} column of 5-μm beads. Gradient programs used for different solvents are shown in Table II (see System A), and the results are shown in Fig. 6. Although the use of methanol required a longer analysis time (18 min) as opposed to acetonitrile (15 min) and THF (13 min), the nucleosides were best resolved with methanol in the mobile phase. With THF, considerable baseline drift was observed and several components were poorly resolved. Methanol was selected as the organic modifier of the eluent for further studies. Deoxyribonucleosides and ddNs having more lipophilic character, such as azT and purine derivatives, required higher methanol contents for their elution from the column.

Effect of pH in the mobile phase. The difference in the dissociation constants of adenosine (3.8) and cytidine (4.3) derivatives could be exploited under acidic elution conditions. To induce strong positive charges on dN and ddN derivatives for ion-pair chromatographic separations, we used more acidic eluents (pH 2.5–3.0). However, in a separate study we observed that these compounds in HPLC are best resolved at pH 5.5. A decrease in pH causes loss in resolution between dC and 5-methyldeoxycytidine (m^5dC). An increase in pH at first failed to show any effect, but at pH $\geq$ 9 the dissociation constants of inosine (8.8) and thymidine (9.8) derivatives exhibited an anionic character. However,

TABLE I

SPECTRAL PROPERTIES OF DEOXY- AND DIDEOXYRIBONUCLEOSIDES

Nucleoside	Acidic spectrum				Neutral spectrum				Basic spectrum			
	pH	Max. absorb. (nm)	ε_M ($\times 10^{-3}$) (l mol^{-1} cm^{-1})	Min. absorb. (nm)	pH	Max. absorb. (nm)	ε_M ($\times 10^{-3}$) (l mol^{-1} cm^{-1})	Min. absorb. (nm)	pH	Max. absorb. (nm)	ε_M ($\times 10^{-3}$) (l mol^{-1} cm^{-1})	Min. absorb. (nm)
dA	1.0	257.4	14.5	228.6	5.5	269.6	15.2	226.2	12.0	261.2	14.9	232.8
ddA (Fig. 1)	1.0	262.0	nd[a]	229.0	5.5	260.0	nd	228.5	12.0	268.2	nd	237.0
dC	1.0	279.6	13.2	240.8	5.5	270.6	9.0	249.6	12.0	271.6	9.0	249.6
ddC (Fig. 2)	1.0	279.4	nd	241.5	5.5	272.0	nd	249.0	12.0	271.6	nd	249.5
dT	1.0	267.0	9.7	235.0	7.0	267.0	9.7	235.0	12.0	267.0	7.4	246.0
azT (Fig. 3)	1.0	266.6	nd	235.0	5.5	266.4	nd	235.0	12.0	266.4	nd	246.0
d4T (Fig. 4)	1.0	266.2	nd	235.0	5.5	265.5	nd	235.0	12.0	267.0	nd	246.0
dI	3.0	248.0	12.2	223.0	6.0	248.5	12.3	223.0	11.0	253.0	13.1	224.0
ddI (Fig. 5)	1.0	247.5	nd	217.2	5.5	249.2	nd	223.4	12.0	261.2	nd	234.0

[a] nd, Not determined.

TABLE II

GRADIENT ELUTION SYSTEMS USED FOR HPLC SEPARATIONS

System	Stage	Time (min)	Flow-rate (ml/min)	Gradient mode	Elution solvent (%)					
					With methanol		With acetonitrile		With THF	
					Buffer[a]	Methanol	Buffer	Acetonitrile	Buffer	THF
A	Equilibration	10	1.0	Step	95	5	97	3	98	2
	Start	3	1.0	Linear	80	20	86	14	90	10
		5	1.0	Linear	70	30	79	21	83	17
		4	1.0	Linear	40	60	51	49	64	36
		4	1.0	Linear	5	95	7	93	33	67
	Hold	3	1.0	Step	5	95	7	93	33	67
	Regeneration	5	1.0	Linear	95	5	97	3	98	2
					Buffer–OSA[b]	Methanol				
B	Equilibration	10	1.0	Step	85	15				
	Start	20	1.0	Linear	50	50				
		1	1.0	Hold	50	50				
	Regeneration	3	1.0	Linear	5	95				
		5	1.0	Linear	95	5				
					Buffer–OSA[b]	Methanol				
C	Equilibration	10	1.2	Step	95	5				
	Start	3	1.2	Linear	80	20				
		5	1.2	Linear	70	30				
		4	1.2	Linear	40	60				
		4	1.2	Linear	5	95				
	Regeneration	3	1.2	Step	5	95				
		5	1.2	Linear	95	5				
					Buffer[c]	Methanol				
D	Equilibration	10	1.0	Step	95	5				
	Start	3	1.0	Linear	80	20				
		5	1.0	Linear	70	30				
		4	1.0	Linear	40	60				
		4	1.0	Linear	5	95				
	Regeneration	3	1.0	Step	5	95				
		5	1.0	Linear	95	5				

[a] 20 m*M* sodium succinate–succinic acid buffer with 1 m*M* sodium azide (pH 5.5).
[b] 20 m*M* sodium citrate–citric acid buffer with 1 m*M* OSA, 0.2 m*M* EDTA, 1 m*M* sodium azide (pH 3.0).
[c] 50 m*M* sodium phosphate–phosphoric acid buffer with 1 m*M* sodium azide (pH 2.5).

such a highly alkaline solution was unacceptable for the stability of the column. An eluent of pH 5.5 was selected for these studies.

Effect of column temperature. An increase in the column temperature enhanced the elution of purine derivatives more than those of the pyrimidines. Similarly, the elution of ddN derivatives was enhanced more than those of their parent nucleosides (results not shown). An increase in column temperature significantly lowered the elution time, but not without a substantial decrease in the resolution of early-eluting peaks, such as dC, m^5dC, dG, and

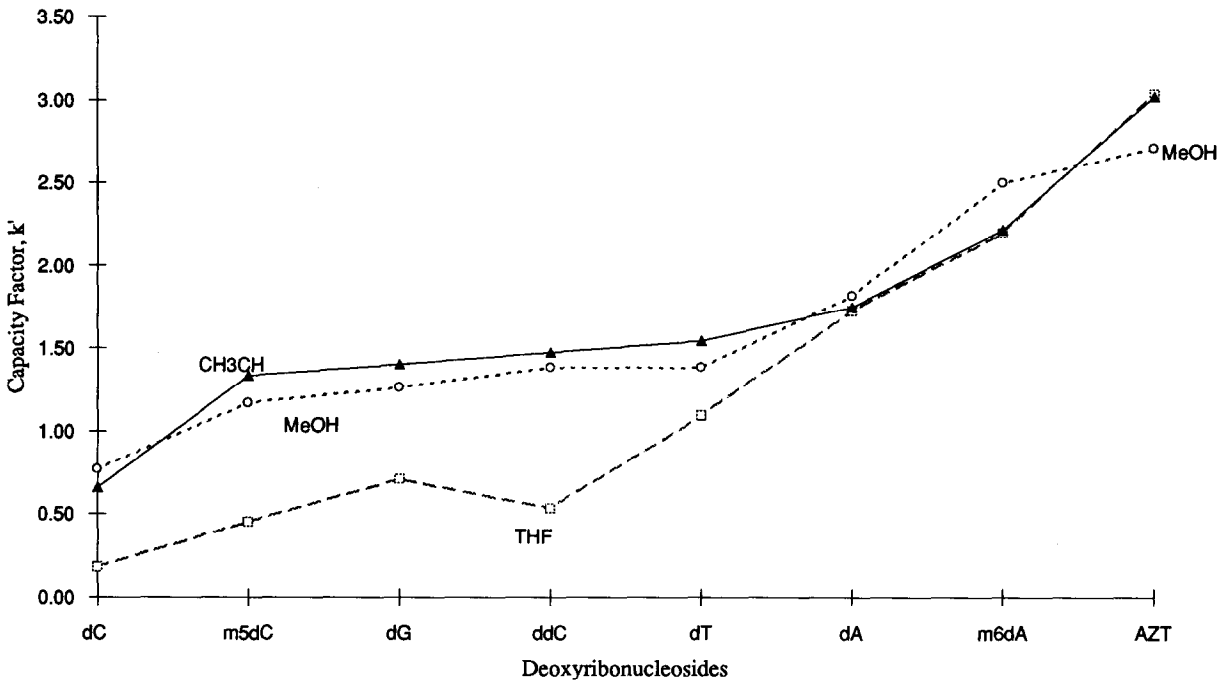

Fig. 6. Effect of organic modifiers [acetonitrile (CH_3CN), methanol (MeOH) and tetrahydrofuran (THF)] on the separation of nucleoside derivatives in HPLC.

ddC components of the mixture. For this reason and ease of operation, room temperature was used for subsequent work.

Separation by ion-pair chromatography. An ion-pairing agent, 1 m*M* OSA, was added to the elution buffer, 20 m*M* sodium citrate–citric acid (pH 3.0). Two isocratic elution methods were studied for the separation of a mixture of ddNs (ddA, ddC, ddI, d4T, and azT). A mixture of 95% buffer–OSA and 5% methanol failed to elute the nucleosides from the column. A 10% increase in the methanol content, *i.e.*, using 85% buffer–OSA and 15% methanol, eluted four of the five components of the mixture. The method was then modified to a gradient elution and two different gradient schemes were examined. The first gradient system (B in Table II) produced overlapping peaks of ddC and ddI, followed by a pure peak of d4T, and finally the last two components (ddA and azT) eluted as a mixed peak. The second gradient (C in Table II), although it yielded four sharp peaks in 17 min, it failed to resolve the fifth component (azT) and had a very erratic baseline. To improve the resolution, a gradient (D in Table II) of lower pH (50 m*M* sodium phosphate buffer with 1 m*M* OSA, pH 2.5) and also an isocratic system (85% buffer–14% methanol) were tried. Again, only four of the five components could be resolved and the first two peaks were badly overlapped. From these results, the ion-pairing mode did not appear to offer any special advantage for the separation of these compounds. Several gradient systems involving no use of the ion-pairing agent were examined by systematically changing the methanol concentration. From these results, it was concluded that the separation of ddC from ddI cannot be achieved simply by altering the organic modifier in the gradient system.

Separation on different reversed-phase columns. Reversed-phase C_{18} columns (25 cm × 0.45 cm I.D.) of 5-μm and 10-μm beads from different manufacturers were examined for the separation of a mixture of five nucleoside analogs (ddA, ddC, ddI, d4T, and azT) using gradient elution (System D in Table II). The results (capacity factors) are given in

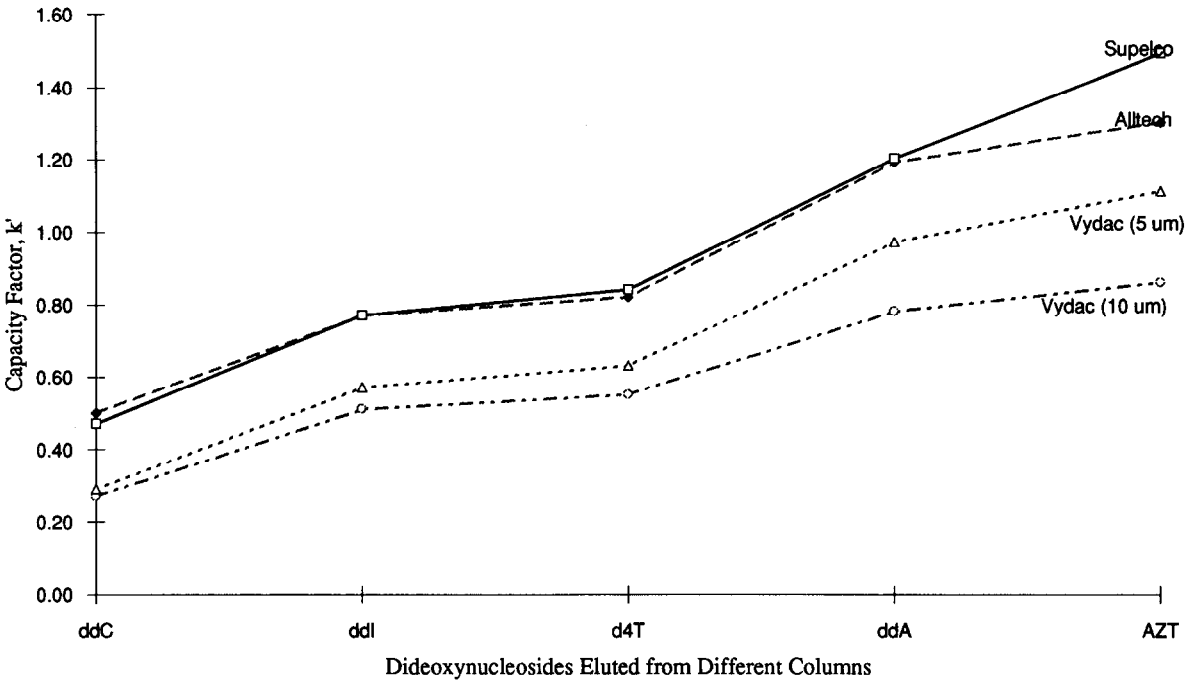

Fig. 7. Performance of different reversed-phase columns in the separation of dideoxyribonucleoside derivatives in HPLC.

Fig. 7. The two Vydac columns, with 5- and 10-μm beads, gave poor resolutions in general and showed little separation between ddI and d4T components. The Supelco and Alltech columns produced larger k' values, but the former column resolved ddI more satisfactorily from d4T, and similarly ddA from azT. Whereas analysis with the Vydac columns required 18–20 min, separations were completed in less than 17 min with the other two columns. The Alltech column caused trailing of the peaks. The best and fastest separations were obtained with the use of Supelco columns.

Representative separations. A typical separation of a mixture of four common dNs and two minor components of DNAs (m^5dC and m^6dA) with the Supelco column and gradient elution System C (Table II, pH 5.5 buffer) is shown in Fig. 8. Under these conditions, the analysis is completed in 15.5 min and the baseline remains fairly stable. A DNA sample contains either m^5dC in prokaryotes or m^6dA in eukaryotes in addition to the common dNs. Thus, the analysis of a prokaryotic DNA sample is completed in less than 13 min. The analysis of the eukaryotic DNA sample can be reduced significantly by modifying elution System A, *i.e.*, by starting elution with 30% methanol and using a step gradient after 60% methanol in the system.

A typical separation of a mixture of five ddNs under conditions identical with those for dNs separation (see above) is shown in Fig. 9. A complex mixture of both six ddNs and six dNs was also separated under these conditions. Although all peaks were resolved satisfactorily for quantification, the separation among m^5dC, ddC and dG did not exceed a resolution factor of 1.0 [36]. No baseline drift at 260 nm was observed, but a small drift was noted in the eluent monitored at 280 nm.

A representative separation of a normal human plasma sample spiked with five ddNs is shown in Fig. 10. No interference is observed between several UV-absorbing plasma proteins and various ddNs, including dNs (results not shown). The plasma proteins are separated into three distinct peaks. Whereas the first two peaks eluted immediately after the void volume in a ratio of 1:1.4, the third peak, eluting in 4 min, varied in concentration from one plas-

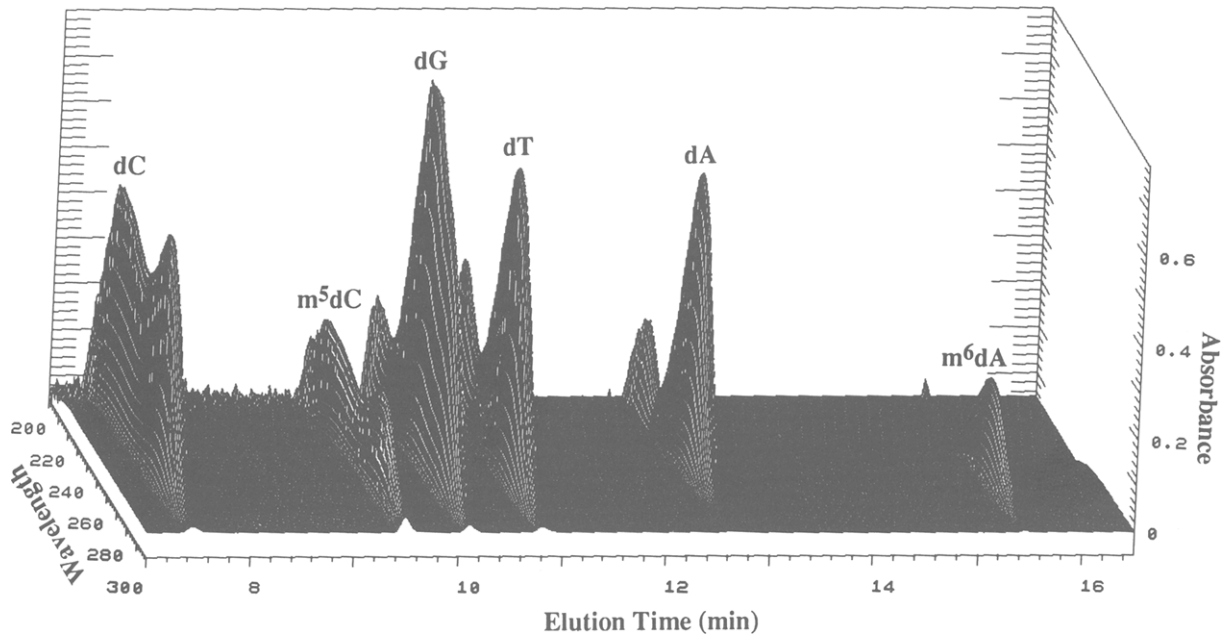

Fig. 8. HPLC: separation of a mixture of the common deoxyribonucleosides and two minor components of DNA (see Results for details).

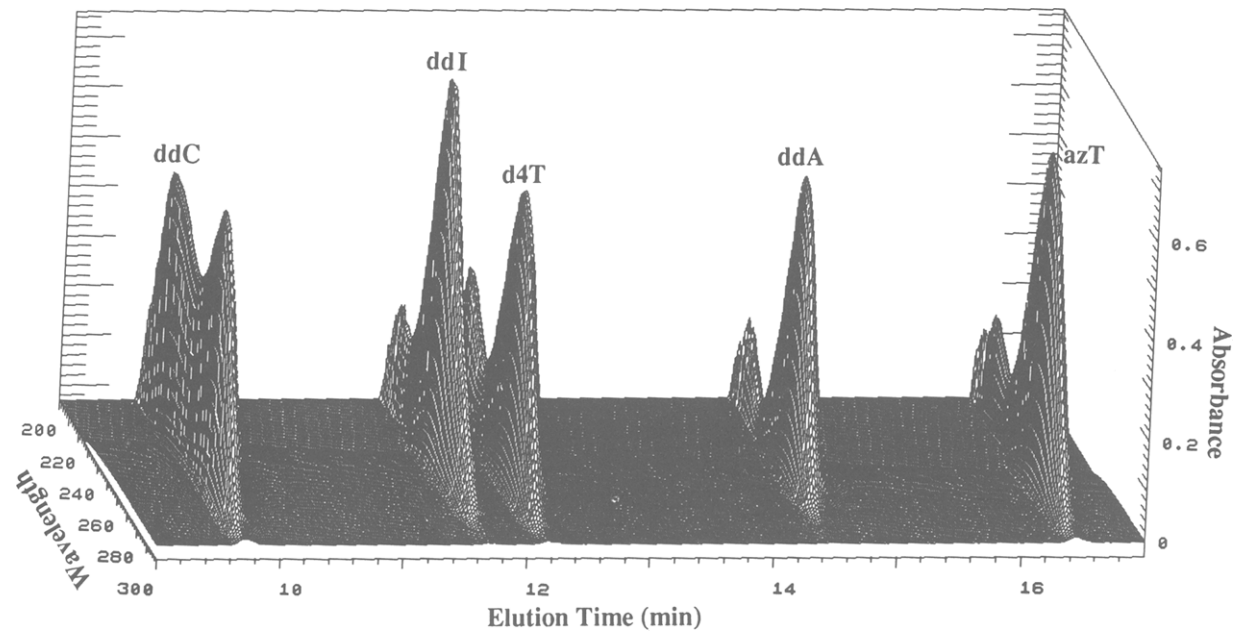

Fig. 9. HPLC: typical separation of a mixture of six dideoxyribonucleoside derivatives (see Results for details).

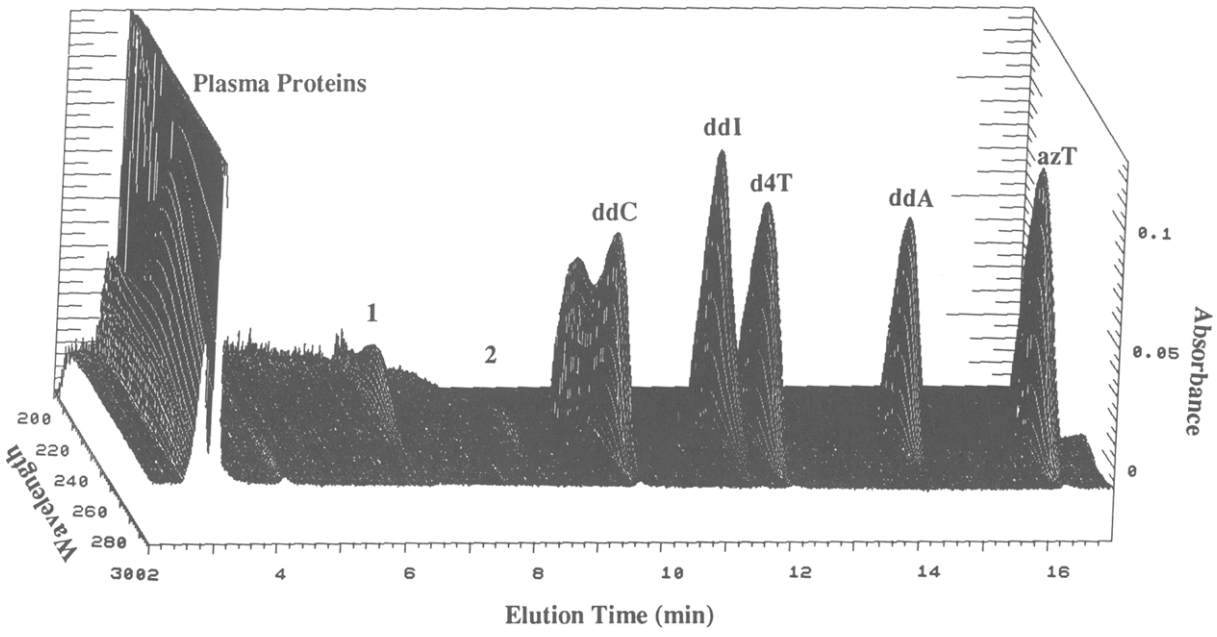

Fig. 10. HPLC: separation of a human blood plasma sample spiked with a mixture of six dideoxyribonucleoside derivatives (see Results for details).

ma donor to another, but never exceeded 10% of the other two peaks. Two contaminants (peak 1, 6.2 min, and peak 2, 8.2 min) elute between the plasma protein peaks and ddC; they are derived from ddNs and mostly from the ddI sample. The analysis is completed in 16 min. Numerous analyses of ddN mixtures have given identical results with no loss of resolution and repeated use of the same column, provided that appropriate safeguards are observed.

Development of a micellar electrokinetic capillary electrophoresis method

The separation of the ddNs and their derivatives could not be achieved by classical CE, *i.e.*, merely by electroosmosis, because these compounds hardly exhibit any charge difference. For this reason, MEKC was employed to resolve these compounds. To create micelles in the capillary columns, several surfactants in different buffers were examined. Cationic surfactants, such as tetrabutylammonium dihydrogenphosphate (TBAP) and dodecyltrimethylammonium bromide (DTAB) gave no separation of the ddN derivatives. This can be explained by the observation that the strong cationic group (N^+) of these surfactants initially diminishes the electroosmotic force by furnishing counter charge to the capillary surface (silanol groups). A higher concentration of cationic surfactants caused a reversal of the electroosmotic (direction) flow and the sample solutes migrated towards the sample-end electrode. Peaks could be monitored only when the electrode polarity was altered, *i.e.*, sample-end electrode made cathode and the monitor-end electrode made anode. The ddN mixture eluted as the first peak of Glo-azT in the breakthrough (14 min) and thereafter all five ddNs derivatives co-eluted in 21 min. The reversal of the elution sequence and the decrease in resolution are caused by the charge reversal of the double layer at the capillary surface. However, satisfactory results were obtained by using an anionic surfactant, such as sodium dodecyl sulphate (SDS). A typical separation of a mixture of representative ddN derivatives is shown in Fig. 11 and Table III. The front peak eluting in 7.4 min was derived from contaminants, mostly present in the ddI sample. In contrast to HPLC, d4T and azT components elute much earlier in MEKC.

TABLE III

ANALYSIS OF A MIXTURE OF DIDEOXYNUCLEOSIDE DERIVATIVES BY MEKC

Peak No.	Dideoxynucleoside derivative	Retention time (min)	Absorbance at 260 nm	Amount (pmol)
1	Contaminant	7.4	0.14	0.73
2	d4T	7.7	0.19	0.89
3	ddC	7.9	0.16	0.84
4	ddI	8.4	0.10	0.52
5	azT	8.6	0.18	0.85
6	ddA	8.8	0.20	0.61
7	Glo-azT	9.9	0.18	0.80
Total		10	1.15	5.24

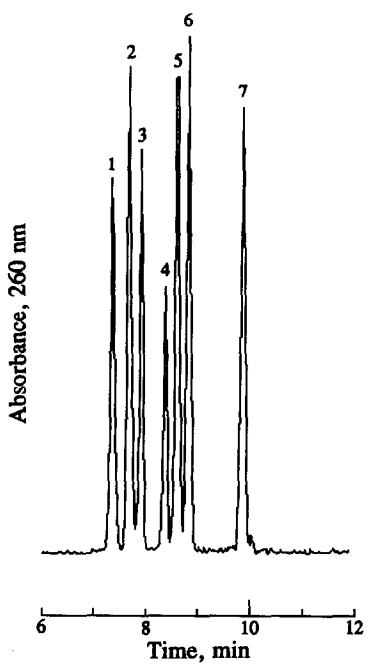

Fig. 11. An artificial mixture of five dideoxynucleosides and Glo-azT derivative was applied (5 s, 3.0 nl) to a fused-silica capillary column (52 cm × 75 μm I.D.) and electrophoresis was carried out in 50 m*M* phosphate buffer containing 40 m*M* SDS (pH 6.5). The separations were performed at 20 kV (116 μA) at 21 ± 0.5°C and the liquid flow was monitored at 260 nm. The front peak was derived from impurities present in the synthetic ddI sample.

DISCUSSION

Development of HPLC methods for the separation of dideoxyribonucleosides

One or more of ddN derivatives have been separated using HPLC mostly with isocratic elutions [2,5–20] and rarely with a gradient elution system [3]. Almost all investigators have used a C_{18} reversed-phase column (25 × 0.46 cm). Although mostly acetonitrile (in buffers of pH 6.8–7.0) has been used in an isocratic elution, DMF [6] and pH 2.7 [5] have also been employed. No systematic study has been carried out with regard to the use of isocratic *versus* gradient elution, the effect of different organic modifiers, ion-pair chromatography and the performance of reversed-phase columns from one manufacturer to the other.

A method for the separation of five selected ddN derivatives was studied here in detail. These compounds are either used currently (azT, ddI and ddC) or are under human trials (d4T and ddA) for treatment of HIV. The results of this study demonstrate the separation properties of these important nucleoside analogs in a systematic manner. Also included in this study were the separation of the common dNs because these compounds are expected to co-elute with their analogs because of similarities in their structures. The spectral properties of ddNs

were also compared with those of their parent derivatives because these compounds are detected in HPLC by UV absorption.

Spectral properties of dideoxyribonucleosides

The results indicate that the spectra of several ddNs (azT, d4T and ddC) resemble mostly those of their parent dNs because the dissociation constants do not appear to change with modifications in the sugar moiety of the nucleoside. However, ddA spectra are an exception to this rule and they differ from those of dA. While red shifts (towards lower energy) are noted at pH 1 and 12, a blue shift (towards higher energy) is observed at pH 5.5. Similarly, the ddI spectrum, as compared with the dA spectrum, exhibits a red shift under alkaline condition, but none under acidic conditions and very little at pH 5.5. Apparently the lack of an ionizable hydroxyl group in the 3′-position contributes to this difference in the spectrum of ddI (pK_a of 3′-OH = 12). However, spectral differences between ddA and dA cannot be explained on the basis of pK_a differences, since the dissociation constants of the two molecules are identical. Interestingly, the spectra of d4T and azT molecules resemble very closely those of dT, although the deoxyribose is extensively modified in the two structures.

Study of HPLC parameters

Isocratic elution offers simplicity of operation, but this method could not resolve components of the mixture. The effect of organic modifiers on the elution behavior of these compounds clearly indicates that methanol is the modifier of choice (see Table II and Fig. 6). A detailed study of the separation by ion-pair chromatography indicates that the ion-pairing agent (OSA), even under different elution conditions (solvent composition and pH), did not enhance the separation of these molecules. Hydrophobic interactions between the C_8-chain alkyl groups of the agent and the C_{18} alkyl chains of the column present N^+ surface charge for ion pairing with any counter ion (OH) of the nucleosides. As pentose hydroxyls are not ionized unless in the vicinity of pH 12, only heterocyclic ring enols can contribute the counter charge. However, these enols are not ionized under the separation conditions employed. It is therefore not surprising to observe that the ion-pairing agent fails to the improve resolution of these compounds, which exhibit similar characteristics.

The bead size and the bead shape of the column matrix, as indicated in earlier studies [37], also appear to be important for these separations. The uniform spherical beads and those of the smaller diameter (5 *vs.* 10 μm) produced superior resolutions, *e.g.*, less peak tailing and greater peak height-to-width ratio [36]. Reversed-phase columns with alkyl chains smaller than octadecyl, such as C_4 and C_8, did not offer hydrophobic interactions necessary for these separations (results not shown). The study of the reversed-phase C_{18} columns from three manufacturers reveals that they produce different separations under identical conditions. Each column was described to have a column matrix of similar dimensions and properties. The difference in separation displayed by the different columns can therefore be explained only on the basis of actual uniformity of the beads and capping of the residual silanol groups.

HPLC method of choice

The selected separation method not only demonstrates the resolution of the five ddN derivatives but also those of the six common dNs. The method in addition allows the separation of these compounds even in the presence of blood plasma proteins. Although the metabolic end products of the ddN derivatives (*i.e.*, ddN phosphates) were not included owing to their unavailability, these compounds are expected to behave differently and elute far away from the parent drug. For example, Glo-azT elutes last and away from azT when a mixture of ddNs and this compound is applied to the column (results not shown). The presence of polyols (glucuronide) in the structure makes the molecule slightly more hydrophilic, but the addition of glucuronide to the drug molecule promotes its elution later than the parent compound, azT.

Development of a micellar electrokinetic capillary electrophoresis method

Amphiphilic surfactants, such as SDS, tend to form water-dispersed structurally ordered micelles, which are globules of numerous surfactants arranged with their hydrophilic charged groups on the surface and the hydrophobic tail in the interior of micelles. The micelles are formed when a critical

concentration of the surfactant is reached in the aqueous phase. The separation in MEKC depends on the partition of solutes between the micelles and the aqueous phase. In this study, classical CE did not affect the separation of dN or ddN derivatives. The use of cationic surfactants, such as TBAP and DTAB, propelled ddNs towards the sample-end electrode and resulted in no separations (see Results for details). When SDS was used as the anionic surfactant, MEKC resolved all seven components of the ddN mixture in *ca.* 3 min, although a breakthrough peak appeared in 7.4 min (Fig. 11). This compares very favorably with HPLC separations of 16.5 min (Fig. 10).

Factors responsible for the separation of solutes in MEKC include their partitioning between the hydrophobic interior of the micelles and the polar aqueous phase, electroosmotic flow of the aqueous phase and electrophoretic migration of the micelles. Of these three factors, electroosmotic flow is the prominent force in determining the direction of flow in MEKC. The bulky micelles migrate at a slower rate than the polar buffer medium. Solutes having greater hydrophobic properties interact more strongly with the micelles than with the buffer, hence they exhibit a slower movement than those solutes having greater hydrophilic properties.

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