Journal of Chromatography, 590 (1992) 153–161
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2395

Isolation of experimental anti-AIDS glycerophospholipids by micro-preparative reversed-phase high-performance liquid chromatography

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ABSTRACT

The experimental anti-AIDS glycerophosphatidic acid: nucleoside (*sn*-1/*sn*-2 diacylglycerol:dideoxynucleotide) drugs 3′-azido-3′-deoxythymidine monophosphate diglyceride (AZT-MP-DG) and 2′,3′-dideoxycytidine monophosphate diglyceride (ddC-MP-DG) were isolated and purified by reversed-phase high-performance liquid chromatography (HPLC). The chromatographic separation was based on the glycerophospholipid moiety of the drugs and detection of the nucleoside component. The separations were optimized on method development columns packed with the stationary phase to be used in the micro-preparative column and monitored by a UV detector. Fractions were collected and analyzed for purity by analytical-scale HPLC and by thin-layer chromatography (TLC). The purity of the recovered drugs based on UV and light-scattering detection and on TLC was greater than 99%. The purified compounds were isolated for studies on structure confirmation, physical, biophysical and formulation properties and anti-HIV efficacy in culture.

INTRODUCTION

In the treatment of acquired immunodeficiency syndrome (AIDS), AIDS-related complex (ARC) and early human immunodeficiency virus (HIV) infection, chemotherapeutic agents are designed to attack one or more stages of the replicative cycle of the HIV [1–4]. An anti-retroviral analogue of thymidine, 3′-azido-2′,3′-dideoxythymidine (azidothymidine, AZT), which inhibits reverse transcription (polymerase), is currently the only drug approved by the US Food and Drug administration (FDA) for the treatment of AIDS/ARC and early asymptomatic HIV infection [5]. However, AZT and other dideoxynucleosides (*e.g.*, ddC and ddI) exhibit dose-limiting toxicity and have relatively short circulating lifetimes [6,7]. In an effort to increase serum half-lives and decreased toxicity and consequently to increase efficacy, a new group of experimental liponucleotide anti-AIDS drugs, originating from earlier work on anti-cancer liponucleotides [8–11], have recently been synthesized [12,13]. These drugs include 16:0/18:1ω9 (*sn*-1/*sn*-2) phosphatidic acid:dideoxynucleoside or diacylglycerol:dideoxynucleotide conjugates, AZT monophosphate diglyceride, (AZT-MP-DG) and dideoxycytidine monophosphate diglyceride (ddC-MP-DG) (Fig. 1) [12,13]. To isolate and purify enough material for molecular confirmation, biophysical and anti-HIV (in culture) studies, a micro-preparative reversed-phase high-performance liquid chromatographic (RP-HPLC) separation was developed.

Chromatographic purification techniques of naturally occurring liponucleotides have been accomplished by thin-layer chromatography (TLC), normal-phase column chromatography on a silica support and ion-exchange column chromatography on silica modified with diethylaminoethyl (DEAE) moieties [14–16]. Synthetic liponucleotides have been purified by TLC and adsorption chromatogra-

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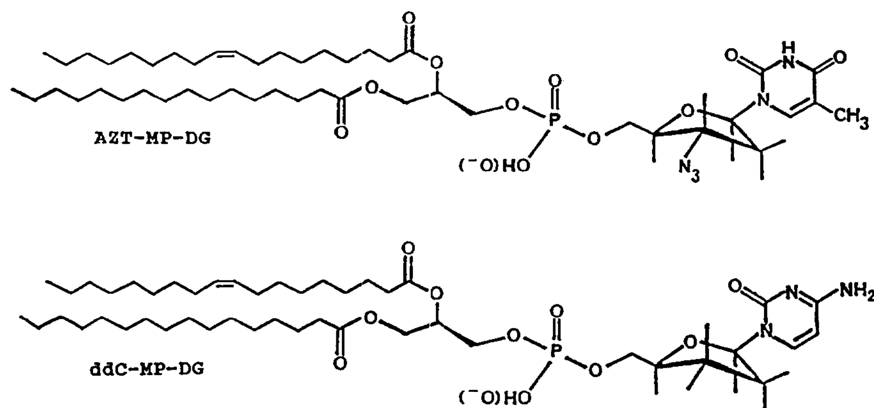

Fig. 1. Structures of AZT-MP-DG and ddC-MP-DG.

phy [17–19] and DEAE chromatography [18,19].

Solvents used to elute the liponucleotides usually contain large amounts of chloroform. As this halogenated solvent is a carcinogen, it is preferable not to use it for the preparative isolation and purification of a drug. In addition, chloroform precludes monitoring by ultraviolet (UV) detection at wavelengths less than 240 nm. As phosphatic acid and other possible contaminants of the synthetic mixture absorb at wavelengths below 210 nm, UV-transparent solvents must be used. To eliminate the use of chloroform, which is often used with silica columns to purify compounds, an RP-HPLC method was developed utilizing a UV-transparent solvent system. The solutes were monitored in the wavelength range 260–280 nm and in the low end of the UV range. In addition, an analytical HPLC method with UV spectral characterization was applied to determine the purity of the fractions [20].

EXPERIMENTAL

Sample preparation

AZT-MP-DG. AZT-MP-DG in the synthetic reaction mixture was first separated from the bulk of starting materials and side-products by silica gel column chromatography. A solution containing *ca.* 50 $\mu g/\mu l$ of AZT-MP-DG was prepared in chloroform–methanol (8:2) with 10 μl of 0.01% butylated hydroxytoluene (BHT) in methanol added as an antioxidant.

ddC-MP-DG. The gross impurities were separated from ddC-MP-DG in the synthetic reaction mixtures by adsorption HPLC [18]. The fractions were collected and pooled. The pooled fractions were taken to dryness with a stream of nitrogen, leaving *ca.* 100 mg of a clear, waxy solid. To dissolve the solid, 2 ml of chloroform–methanol (3:2) were used together with 10 μl of 0.01% BHT in methanol. The concentration of the working solution was *ca.* 50 $\mu g/\mu l$.

Materials

All solvents and reagents (Fisher Scientific, Pittsburgh, PA, USA) for the method development, micro-preparative separations and analysis were of HPLC grade. Water was doubly distilled and deionized. Each solvent was filtered through a 0.45-μm nylon-66 filter (AllTech, Deerfield, IL, USA). The mobile phases contained 1–1.4 mM KH_2PO_4 adjusted to pH 2.4 with phosphoric acid.

Method development

The chromatographic system used for the method development studies consisted of a Model M6000A pump (Waters–Millipore, Milford, MA, USA), a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 100 μl loop and a Knauer variable-wavelength UV detector (Sonntek, Woodclift Lake, NJ, USA). Method development columns were 25 × 0.46 cm I.D., packed with YMC Prep-10 C_{18} (10-μm) silica (Yamamura Chemical, Kyoto, Japan). The columns were packed using a Haskel (Burbank, CA, USA) Pump. The separa-

tions were optimized on a small scale, thus reducing solvent and sample consumption.

The wavelengths used to monitor the separations were 267 nm for AZT-MP-DG and 280 nm for ddC-MP-DG. Absorption at these wavelengths was due to the pyrimidine moieties.

The mobile phase consisted of methanol–1.4 mM KH_2PO_4 (93:7, v/v) at pH 2.4 for AZT-MP-DG and methanol–1 mM KH_2PO_4 (95:5, v/v) at pH 2.4 for ddC-MP-DG. After elution of the liponucleotides, the columns were flushed with 100% methanol to elute any strongly retained impurities. After the highly retained solutes had been washed off with methanol, the column was re-equilibrated with the mobile phase. The optimum method development flow-rate was 2.8 ml/min for AZT-MP-DG and 3.5 ml/min for ddC-MP-DG. All separations were achieved at room temperature. The chromatograms were recorded on an HP 3394A integrator (Hewlett-Packard, Avondale, PA, USA) at 0.2 cm/min and an Omniscribe recorder (Houston Instruments, Austin, TX, USA) at 0.25 cm/min.

Micro-preparative HPLC

Micro-preparative separations were carried out on a 25 × 1.0 cm I.D. column packed with YMC Prep-10 C_{18} (10-μm) silica (Yamamura Chemical, Japan). The HPLC system was a SepTech (Wakefield, RI, USA) NovaPrep 5000 operated at a flow-rate of 10 ml/min for AZT-MP-DG and 15 ml/min for ddC-MP-DG. The system was operated under computer control, utilizing TurboPrep software (SepTech). The variable-wavelength detector was set at 267 nm with a sensitivity of 0.64 a.u.f.s. to monitor AZT-MP-DG and at 280 nm for ddC-MP-DG. The working solution was injected manually with a syringe. The NovaPrep contains two pumps: pump A delivered methanol–KH_2PO_4 and pump B methanol.

Analytical HPLC

The fractions collected during method development and micro-preparative separations were analyzed for purity. The analytical system consisted of a Waters Model M6000 pump, a Rheodyne 10-μl injector, a Schoeffel Spectro Flow Monitor SF 770 variable-wavelength detector (Kratos, Westwood, NJ, USA), a rapid-scanning UV detector (Barspec, Rehovot, Israel) and an evaporative light-scattering detector (Varex, Burtonsville, MD, USA).

For the AZT-MP-DG analysis a 25 × 0.40 cm I.D. LiChrospher 100 C_{18} (5-μm) column (EM Science, Gibbstown, NJ, USA) with methanol–1 mM KH_2PO_4 (pH 2.4) (95:5) as the mobile phase at a flow-rate of 1.5 ml/min was used. A sensitivity of 0.02 a.u.f.s. and wavelengths of 267 and 208 nm [the two absorbance maxima of AZT-MP-DG (Fig. 2a)] were used to monitor the solute. The low wavelength was useful to detect impurities lacking strong UV chromophores such as phosphatidic acid or glycerol.

The ddC-MP-DG was analyzed on a 25 × 0.46 cm I.D. C_{18} (5-μm) column (HPLC-Systeme, Berlin, Germany) with methanol–1 mM KH_2PO_4 (pH 2.4) (95:5) as the mobile phase at a flow-rate of 2 ml/min. To monitor the solute, wavelengths of 280 and 208 nm were used [the two absorbance maxima of ddC-MP-DG (Fig. 2b)].

For the liponucleotides the light-scattering detector was used under the following conditions: nitrogen pressure, 85 mmHg; exhaust temperature, 92.5°C; and heater temperature, 150.0°C. The mobile phase was methanol–1 mM ammonia solution (pH 2.4) (95:5) at a flow-rate of 1.2 ml/min.

Collected and pooled fractions were also analyzed by TLC on silica gel plates (Fisher Scientific). The mobile phase was chloroform–methanol–2-propanol–water–triethylamine (30:9:25:7:25) [21]. For detection, molybdenum blue, which is specific for phosphorus in the glycerophospholipids, and sulfuric acid, which is a general reagent for all organic compounds, were used.

RESULTS AND DISCUSSION

Liponucleotides are analogues of glycerophospholipids. Like naturally occurring glycerophospholipids, they are composed of a polar head group and two non-polar fatty acid chains esterified to the diglyceride moiety. The polar head groups of the experimental drugs AZT-MP-DG and ddC-MP-DG consist of the anti-viral nucleosides (AZT and ddC) coupled to 16:0/18:1ω9 (*sn*-1/*sn*-2) phosphatidic acid (PA) [12,13]. The physical properties of the liponucleotides resemble those of natural glycerophospholipids. Thus separations have been based on glycerophospholipids which are generally separated using the normal-phase mode. However, normal-phase separations for glycerophospholipids

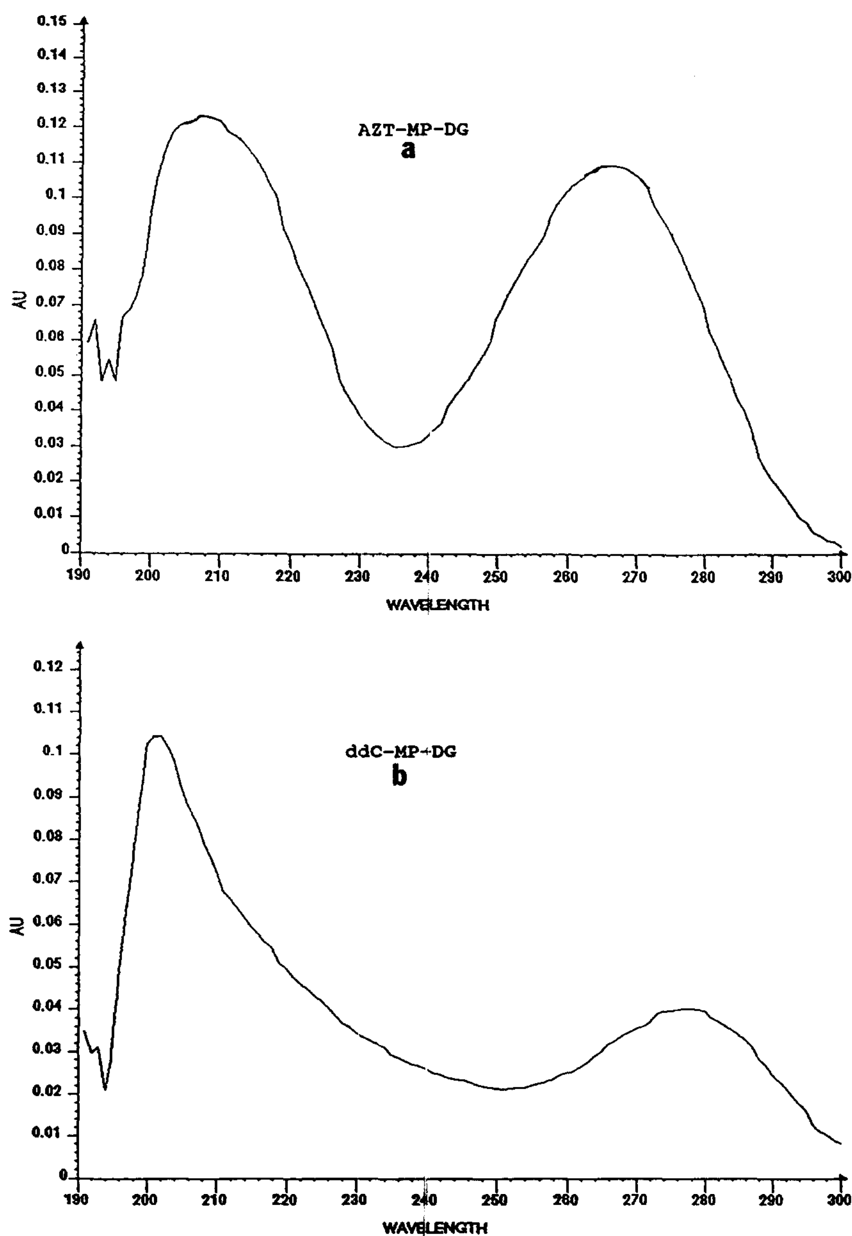

Fig. 2. (a) UV spectrum of purified AZT-MP-DG obtained with the rapid-scanning detector. Scan rate, ten spectra per second. Absorbance maxima: 208 and 267 nm. Analytical chromatographic conditions: mobile phase, methanol–1 mM KH_2PO_4 (pH 2.4) (95:5); flow-rate, 1.5 ml/min. (b) UV spectrum of purified ddC-MP-DG obtained with the rapid-scanning detector. Scan rate, ten spectra per second. Absorbance maxima: 208 and 280 nm. Analytical chromatographic conditions: mobile phase, as in (a); flow-rate, 2 ml/min.

require ternary mobile phases and/or gradient elution whereas with RP-HPLC a binary mobile phase with isocratic elution can be used, which is preferable for preparative separations. Mobile phase compositions for the reversed-phase separation of natural glycerophospholipids typically contain 90–95% methanol and 1–2 mM KH_2PO_4 [22,23]. In addition to KH_2PO_4, ammonium acetate can be

used as the salt in the mobile phase [24]. Ammonium acetate 1 mM gave the same k' values as those obtained with 1 mM KH_2PO_4.

For purity analysis, aliquots of the collected fractions were injected onto the analytical columns. As commercial standards were not available for either compound, the liponucleotide peaks were characterized with UV detection using absorbance ratios and peak-purity measurements [25]. In addition to UV and TLC analysis, a light-scattering detector was used.

AZT-MP-DG

The optimum mobile phase composition for AZT-MP-DG was methanol–1.4 mM KH_2PO_4 (pH 2.4) (93:7). The AZT-MP-DG solution was injected onto the analytical 5-μm column to determine the number of components present (Fig. 3). Impurities eluted near the solvent front and after the AZT-MP-DG peak.

Using the method development column, a loading study was performed to determine the highest load injectable while maintaining a purity level of 99%. Various volumes of the AZT-MP-DG solution were injected and fractions collected. Each fraction was concentrated with a stream of nitrogen to *ca.* 2 ml and analyzed by HPLC. An aliquot of 100 μl of the 50 μg/μl (5 mg) solution was the optimum load.

The separation was scaled up to the micro-preparative column. For the micro-preparative separation a flow-rate of 10 ml/min provided adequate retention and resolution. A 400-μl (20-mg) aliquot was injected (Fig. 4), which produced a split peak. Eighteen 5-ml fractions were collected across the split peak and each was concentrated by rotary vacuum distillation to *ca.* 2 ml. Each fraction was analyzed for purity by analytical HPLC. The k' values of the AZT-MP-DG fractions across the split peak were identical (3.5). Hence the peak splitting may be attributed to the solvent effect due to the chloroform used as the sample diluent. Peak splitting has also been reported at the analytical level for the AZT-MP-DG [20]. Fractions 1 and 2 were discarded because of early-eluting impurities. Fractions 3–17, containing AZT-MP-DG of 99% purity, were

Fig. 3. Analytical HPLC of crude AZT-MP-DG, 5 μl of 50 μg/μl solution (a.u.f.s. 0.04). For chromatographic conditions, see Experimental.

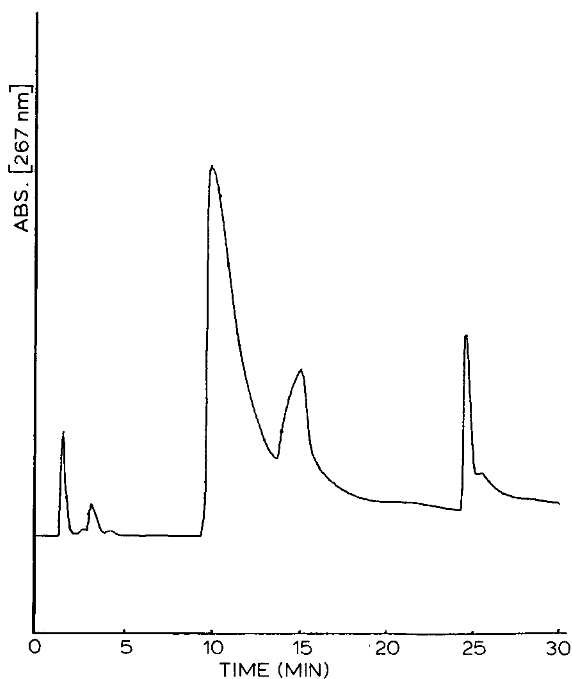

Fig. 4. Micro-preparative chromatogram of 20 mg of AZT-MP-DG. Mobile phase, methanol–1.4 mM KH_2PO_4 (pH 2.4) (93:7); flow-rate, 10 ml/min; 267 nm; 0.64 a.u.f.s. Fraction were collected between 9 and 20 min.

pooled. Fraction 18 contained a very minor amount of the product and an impurity after the product, so it was not added to the pool. The pooled fractions were concentrated to *ca.* 2 ml with rotary vacuum distillation. Fig. 5a illustrates the chromatogram of the pooled fractions. Based on UV detection at 267 nm, the purity of the AZT-MP-DG was 99.8%. The pooled fractions were also monitored at 208 nm to detect any impurities that did not absorb at 267 nm (Fig. 5b). However, no additional impurities were detected at 208 nm. Peak splitting was not observed for the pooled fractions, because the AZT-MP-DG recovered was in a solvent mixture similar to the mobile phase used for the analytical HPLC.

The rapid-scanning UV detector was used to obtain absorbance ratios and peak-purity values. Three wavelengths (267, 220 and 208 nm) were used to determine the ratios. The ratios at 267/220, 267/208 and 220/208 nm were compared to ratios obtained on previously purified AZT-MP-DG. For multiple determinations the absorbance ratios were consistent, as shown in Table I. Peak-purity values were determined by normalizing the UV spectra along three points of the chromatographic peak of interest. Peak-purity values indicate if an impurity is co-eluting with the solute of interest. Low peak-purity values (<1.000) indicate with high confidence that no other component(s) was co-eluted with the solute of interest [26]. A peak-purity value of 0.225 was determined for AZT-MP-DG, which compared favorably with a value of 0.215 determined for a batch of AZT-MP-DG synthesized previously. To determine the mass of AZT-MP-DG recovered, the pooled fractions were evaporated to dryness with a stream of nitrogen and then extracted (three times) with chloroform–methanol (8:2). The extraction solvent was evaporated with nitrogen, leaving 18 mg of purified AZT-MP-DG.

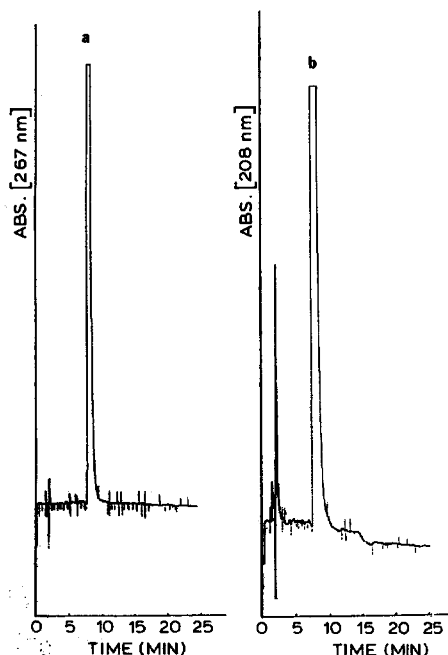

Fig. 5. Analytical HPLC of pooled AZT-MP-DG fractions at (a) 267 and (b) 208 nm (0.02 a.u.f.s.). For chromatographic conditions, see Experimental.

ddC-MP-DG

The ddC-MP-DG mixture was first chromatographed on an analytical column (Fig. 6). In comparison with AZT-MP-DG, more impurities were found. On the method development column an aliquot of 100 μl of the working solution (5 mg) provided the optimum load. During the loading study, the column displayed overload when the k' values decreased with increasing mass injected. The flow-rate was directly scaled to 15 ml/min and 25 mg was the mass injected onto the micro-preparative column. The chromatogram was similar to that in the method development separation (Fig. 7). Nine fractions were collected across the ddC-MP-DG peak. Each fraction was concentrated by rotary vacuum distillation for HPLC analysis.

TABLE I

POOLED AZT-MP-DG FRACTIONS FROM MICRO-PREPARATIVE SEPARATION

	Wavelength ratio		
	267/220 nm	267/208 nm	220/208 nm
	1.22	0.92	0.75
	1.29	0.90	0.70
	1.24	0.86	0.69
Mean:	1.25	0.89	0.71
S.D.:	0.0360	0.03	0.033
R.S.D. (%)[a]:	2.88	3.30	4.58

[a] Relative standard deviation.

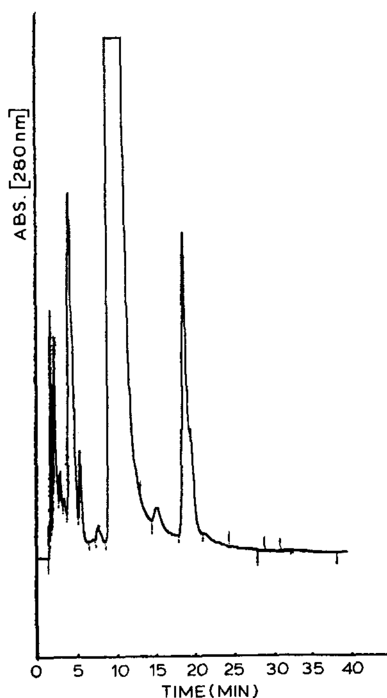

Fig. 6. Analytical HPLC of crude ddC-MP-DG, 5 μl of 50 μg/μl solution (a.u.f.s. 0.04). For chromatographic conditions, see Experimental.

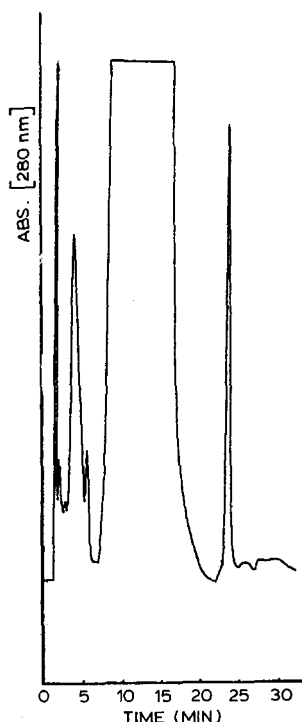

Fig. 7. Micro-preparative chromatogram of 25 mg of ddC-MP-DG. Mobile phase methanol–1 m*M* KH_2PO_4 (pH 2.4) (95:5); flow-rate, 15 ml/min; 280 nm; 0.64 a.u.f.s. Fractions were collected between 7 and 20 min.

The ddC-MP-DG fractions were monitored at 280 and 208 nm and fractions 4–8 were pooled. Fractions 1–3 were not pooled because an impurity eluted prior to the ddC-MP-DG and in fraction 9 an impurity that eluted after ddC-MP-DG was present. As with AZT-MP-DG, monitoring the ddC-MP-DG at 208 nm did not reveal any additional impurities. Representative chromatograms of the pooled fractions at 280 and 208 nm are shown in Fig. 8a and b, respectively. Based on UV absorption, the purity of the pooled ddC-MP-DG was 99.8%. The pooled fractions were also monitored with the rapid-scanning UV detector. Absorbance ratios, listed in Table II, at 280, 220 and 208 nm were determined. The ratios were consistent for multiple injections. A peak purity of 0.326 was obtained for the pooled fractions, indicating that a single component, ddC-MP-DG, was present. To determine the mass of ddC-MP-DG recovered, the pooled fractions were evaporated to dryness with a stream of nitrogen and then extracted (three times) with chloroform–methanol (3:2). The extraction solvent was evaporated with nitrogen, leaving 13.4 mg of purified ddC-MP-DG.

Impurities

Impurities such as phosphatidic acid are difficult to detect with UV detectors even at low wavelengths. Therefore, an evaporative light-scattering detector was used for monitoring compounds that do not possess UV-absorbing chromophores. Pooled fractions of AZT-MP-DG were analyzed with an evaporative light-scattering detector to confirm the purity (Fig. 9) Based on the light-scattering chromatogram, the purity of AZT-MP-DG was greater than 99%.

TLC is very valuable for determining phosphorus-containing compounds selectively by using a molybdenum blue reagent. Universally all carbon-containing compounds were monitored by charring with sulfuric acid. With silica gel thin-layer plates and detection with these two reagents, no impurities

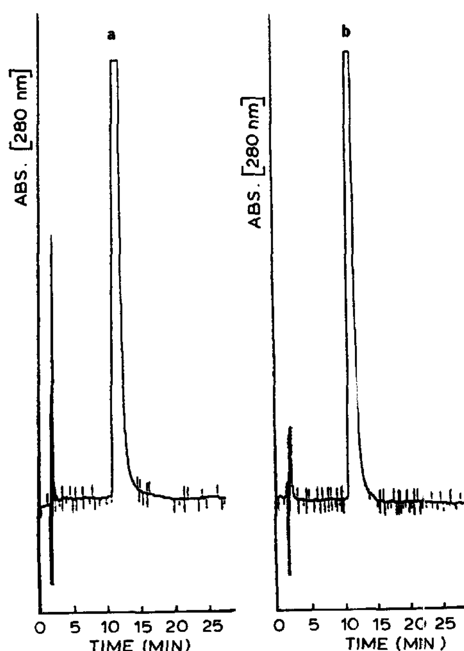

Fig. 8. Analytical HPLC of pooled ddC-MP-DG fractions at (a) 280 and (b) 208 nm (0.02 a.u.f.s.). For chromatographic conditions, see Experimental.

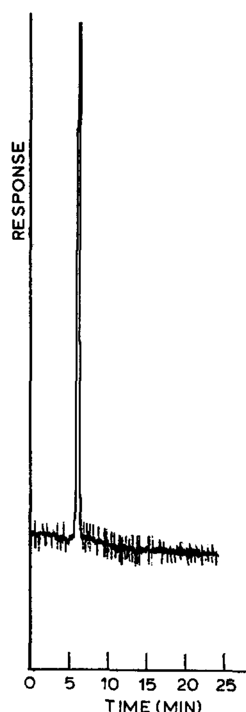

Fig. 9. Evaporative light-scattering chromatogram of pooled AZT-MP-DG fractions. Chromatogrpahic conditions: mobile phase, methanol–1 m*M* ammonia solution (pH 2.4) (95:5); flow-rate, 1.2 ml/min; detector range (sensitivity), 5; heater and exhaust temperatures, 150.0 and 92.5°C, respectively.

were detected in fractions 3–17 of AZT-MP-DG, in fractions 4–8 of ddC-MP-DG or in the pooled fractions of both compounds.

In conclusion, a micro-preparative separation has been developed for the isolation and purification of new experimental anti-HIV glycerophospholipids. The conditions were based on separations of naturally occurring glycerophospholipids on reversed-phase supports and detection based on the nucleotide moiety. The eluent allows for low-UV monitoring to detect impurities that lack strong UV chromophores. AZT-MP-DG and ddC-MP-DG were obtained in purities greater than 99%. The purities were also confirmed using an evaporative light-scattering detector and TLC. The optimized conditions for the micro-preparative column can be adopted with larger preparative columns when greater amounts of the liponucleotides are to be purified.

TABLE II

POOLED ddC-MP-DG FRACTIONS FROM MICRO-PREPARATIVE SEPARATION

	Wavelength ratio		
	280/220 nm	280/208 nm	220/208 nm
	0.62	0.92	0.68
	0.59	0.89	0.66
	0.59	0.89	0.66
Mean:	0.60	0.90	0.67
S.D.	0.016	0.014	0.0072
R.S.D. (%):	2.70	1.58	1.08

ACKNOWLEDGEMENTS

The authors thank SepTech, a Division of EM Industries for the method development columns and YMC for donating the stationary phase and the micro-preparative column. They also thank Varey Corp. for the use of the evaporative light-scattering detector, Barspec Corp. for the rapid-scanning UV detector and Dr. Fred Rabel of EM Science and I.

Molnar of HPLC-Systeme (Berlin, Germany) for the analytical columns. This research was supported in part by a grant from the US National Institute of Allergy and Infectious Diseases (AI 25690) and the National Cooperative Drug Discovery Group for the Treatment fo AIDS (NCDDG/HIV).

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