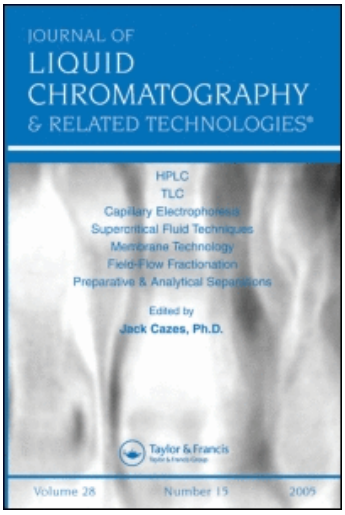

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PREPARATIVE HPLC OF AN EXPERIMENTAL ANTI-HIV ANALOGUE OF AZT: AZIDOTHYMIDINE MONOPHOSPHATE DIGLYCERIDE (AZT-MP-DG)

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ABSTRACT

Monophosphate diglyceride conjugate of AZT containing esterified saturated palmitoyl and unsaturated oleoyl fatty acid, a composition which more closely mimics the natural state (AZT-MP-DG), was synthesized. The purification of AZT-MP-DG from crude synthetic mixture by preparative HPLC is described herein. The practical and rapid chromatographic method developed should be applicable to the purification of structurally related antimetabolic lyponucleotides.

INTRODUCTION

3'-Azido-3'-deoxythymidine (AZT), an antiretroviral analogue of thymidine, is presently the principal drug used for the clinical treatment of

acquired immunodeficiency syndrome (AIDS), AIDS-related-complex (ARC), and early HIV-1 infection. Glycerophospholipid and structurally related conjugates of anti-HIV dideoxynucleosides (ddNs) [e.g., 1-4] or other suitably derivitazable anti-HIV small molecules, can provide a means to substantially improved parent drug efficacy, particularly in view of their (e.g., AZT, ddC, ddI) relatively short plasma half-lives (30-60 minutes) and clinical toxicity. Designed on the basis of the molecular structures, biochemistry, and biophysical properties of natural glycerophospholipid and lysoglycerophospholipid prototypes, such conjugates are potentially superior to antimetabolic ddns or nucleosides in that physical properties such as binding to plasma proteins, capacity to form vesicles, and capacity to deliver and release phosphorylated drug forms, can be afforded [e.g., 1-11]. For biological testing and in animal pharmacological studies, relatively large amounts of a representative member of a class of ddN: glycerophosphatidic acid conjugates, AZT monophosphate diglyceride (AZT-MP-DG), (Fig.1) was required.

CONCLUSION

The glycerophosphatidic acid: nucleoside AZT-MP-DG was purified by normal phase HPLC. Methods development was carried out on a 20 cm x 0.46 cm column, packed with the EM Lichrospher Si 60, 12 μm (E. Merck). The mobile phase was methylene chloride, methanol, and ammonium hydroxide (gradient). A maximum load of 20 mg on a methods column gave a base line

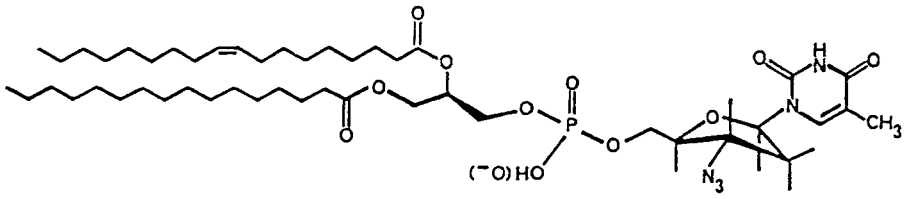

Figure 1. The structure of 3'-azido-3'-deoxythymidine-5'-phosphate diglyceride (C16:0/1ω9,sn-1/sn-2), abbreviated in text as AZT-MP-DG.

separation of AZT-MP-DG. The recovery of AZT-MP-DG from the crude reaction product was quantitative. To analyze the isolated compound, TLC and analytical HPLC (UV detector, 254 nm and a Varex laser light scattering detector) were used. The isolated (AZT-MP-DG) was 99.5% pure by HPLC. The separation was scaled up to a 5.0 x 20 cm, annular expansion (A/E) column (Separations Technology) packed with the same sorbent. The development of a preparative HPLC method for the purification of AZT-MP-DG is described herein, which should be applicable to the separation and purification of other structurally related antimetabolic liponucleotides.

EXPERIMENTAL

The chromatograph used for the methods development consisted of a Varian Vista 5500 HPLC, a Rheodyne 7125 injector, and a Knauer UV detector at 254 nm. The chromatograph used for the larger scale preparative runs was a

Novaprep 5000 Development scale HPLC system (Separations Technology). The data were recorded on a Spectra Physics integrator and then transferred to a personal computer. The HPLC runs were carried out using methylene chloride, methanol and ammonium hydroxide gradient. Each solvent was HPLC grade (OmniSolv, EM Science).

The separations were performed on columns packed at Separations Technology with EM Lichrospher sorbents (E. Merck, Germany). An EM Si 60, 5 μm, 0.40 x 25 cm column was used to analyze the HPLC fractions. A 0.46 x 20 cm column packed with EM Si 60, 10-12 μm sorbent was used for methods development. A 5.0 x 20 cm, SepTech annular expansion (A/E) column was used for scale-up using the same sorbent as in the methods column. All separations were achieved at ambient temperature.

For methods development and loading studies, a 25 mg/ml solution of the crude reaction product in CH_2Cl_2 was used. For the scale-up to a 5.0 x 20 cm column, 1 g/ml solution of the crude reaction mixture in CH_2Cl_2 was used.

Thin layer chromatography (pre-coated TLC plates, Silica 60, E. Merck, Germany) were used to test the purity of the fractions collected from preparative HPLC runs along with analytical HPLC. Methylene chloride/ methanol/ triethyl-amine (80:20:0.5) was used as the TLC developing solvent. The TLC plates were sprayed with Molybdenum blue and charred at 110°C.

TABLE 1 Gradient conditions for the analytical HPLC analysis of AZT-MP-DG.

Time (min)	% A	% B	Flow rate (ml/min)
0	100	0	1
25	0	100	1
26	100	0	1

Solvents: A: CH_2Cl_2/MeOH/NH_4OH (450:50:1)

B: CH_2Cl_2/MeOH/NH_4OH (300:75:5)

Column: EM Lichrospher Silica 60, 5 µm, 0.40 x 25 cm.

Detector: UV 254 nm (Knauer UV Detector) and Laser Light Scattering detector (Varex).

RESULTS AND DISCUSSION

Analytical HPLC:

The gradient conditions for the analytical studies are given in Table 1. Two types of detectors, a Knauer UV detector at 254 nm and a Varex evaporative light scattering detector (ELSD II), were used to monitor the AZT-MP-DG and the impurities in the fractions collected from preparative HPLC runs. The laser light scattering detector does not depend on specific functional group or chromophores; it provides the most precise profile of the character of a given sample, accurately portraying the mass balance. Analysis using the light scattering detector also

Table 2 Gradient conditions for the methods development.

Time (min)	% A	% B	Flow rate (ml/min)
0	100	0	1
25	50	50	1
26	100	0	1

Solvents: A: CH_2Cl_2/MeOH/NH_4OH (450:50:1)

B: CH_2Cl_2/MeOH/NH_4OH (300:75:5)

Column: EM Lichrospher Silica 60, 12 μm, 0.46 x 20 cm.

Detector: UV 254 nm (Knauer UV Detector).

allows impurities to be analysed directly, without a complicated calibration procedure.

Methods Development and loading studies:

Crude yields of dideoxynucleoside mono-phosphate diglycerides typically range from 40-80%. For these studies, AZT-MP-DG obtained in the lower yield range (40%) was used for methods development, loading and preparative HPLC studies. An authentic sample of AZT-MP-DG [16:0-sn-1/18:1-sn-2], whose structure was confirmed by elemental analysis and multinuclear NMR (^1H, ^{13}C, ^{31}P), was employed as an analytical standard. After extensive experimentation, the solvent gradient shown in Table II, and a flow rate of 1 ml/min was found to give

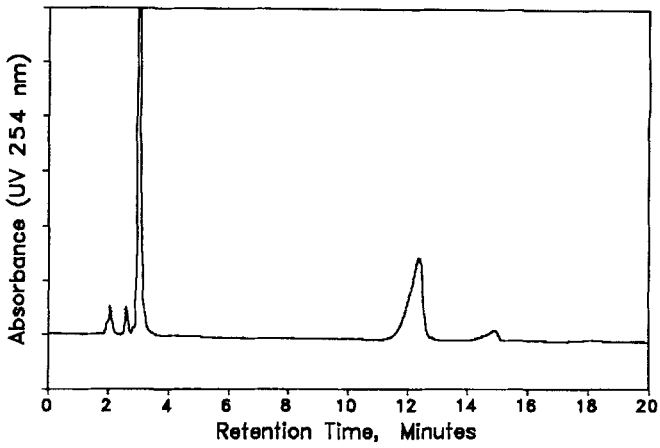

Figure 2. Methods development Chromatogram. Load 20 μg of the crude reaction mixture.

a base-line separation of AZT-MP-DG on the methods column. A representative chromatogram of the crude mixture separation is shown in Figure 2. The load was 20 μg, and the AZT-MP-DG peak was eluted at 12 minutes. The chromatogram from a load of 2 mg of the crude mixture is given in Figure 3.

A maximum load of 20 mg gave a base line separation of the AZT-MP-DG peak, Figure 4. Seven fractions were collected from the AZT-MP-DG peak. The fractions were analyzed by HPLC. Fractions 3-5 collected from 10.8 to 12.6 minutes, contained 99% pure AZT-MP-DG and were combined together. The yield was 40% (by weight of the crude reaction mixture) for the AZT-MP-DG. The recovery of AZT-MP-DG from the crude reaction mixture was 92%.

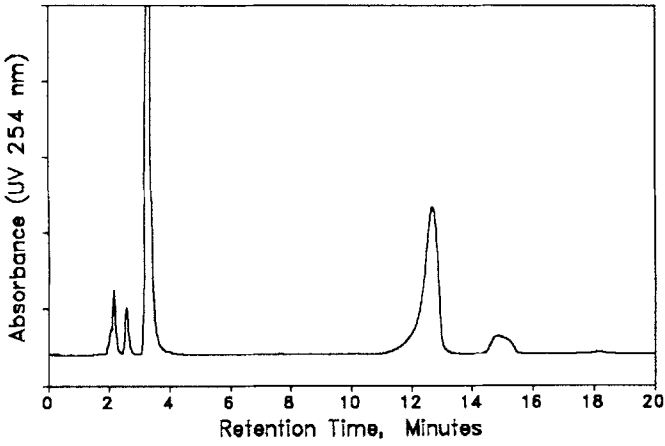

Figure 3. Methods development chromatogram. Load: 2 mg.

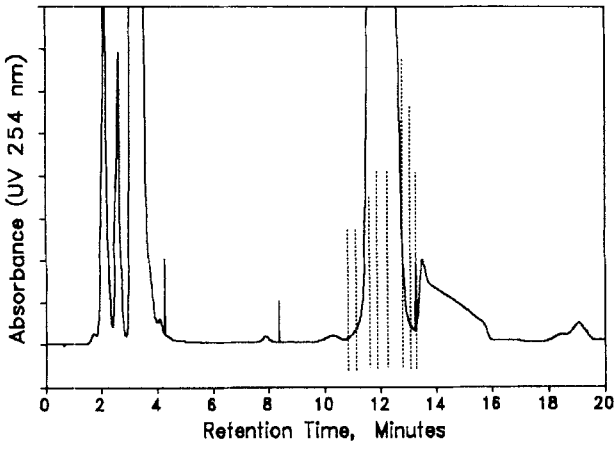

Figure 4. Methods development chromatogram. Load: 20 mg of the crude reaction mixture.

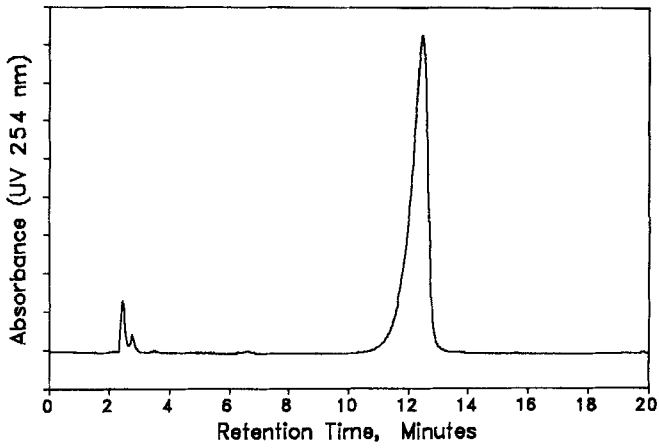

Figure 5. Analytical HPLC of AZT-MP-DG purified on a methods column. Load: 5 µg.

An analytical HPLC chromatogram of the isolated compound is given in Figure 5. The purity of the fractions was also tested by TLC. The product showed a single component on TLC, Rf: 0.65. (methylene chloride/ methanol/ triethyl amine, 80:20:0.5).

SCALE-UP TO PREPARATIVE HPLC [5.0 x 20 cm ANNULAR EXPANSION (A/E)] COLUMN:

The calculated scaleup parameters are shown in Table III. A 2.3 gm sample of the crude reaction product was dissolved in 2.3 ml of CH_2Cl_2 and injected on

Table 3 Calculated scale-up parameters to columns with nominal diameters of 1", 2", and 3".

Column Dia x Length (cm)	Flow rate (ml/min)	Load (g)
0.46 x 20	1.0	0.020
1.93 x 20	17.6	0.230
5.0 x 20	118	2.3
7.5 x 20	256	5.3

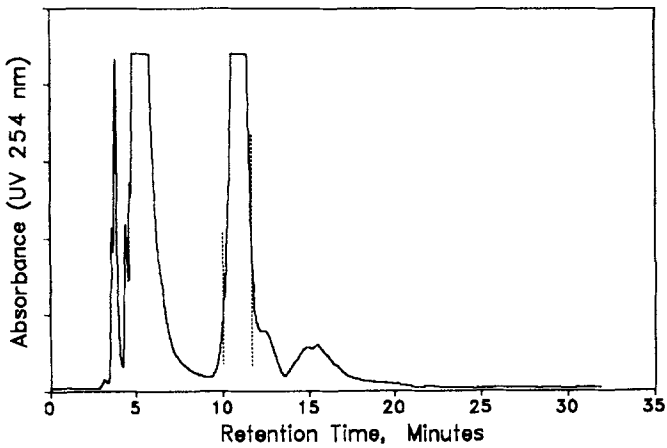

Figure 6. Scaleup to a 5 cm X 20 cm A/E column. Load 2.3 gm.

to the 2" A/E column. The flow rate was 118 ml/min. The solvents and gradient were the same as for the methods development column and are shown in Table II. The prep chromatogram is shown in Figure 6. A 190 ml AZT-MP-DG fraction was collected from 10 min. to 11.8 minutes. The solvent was removed by

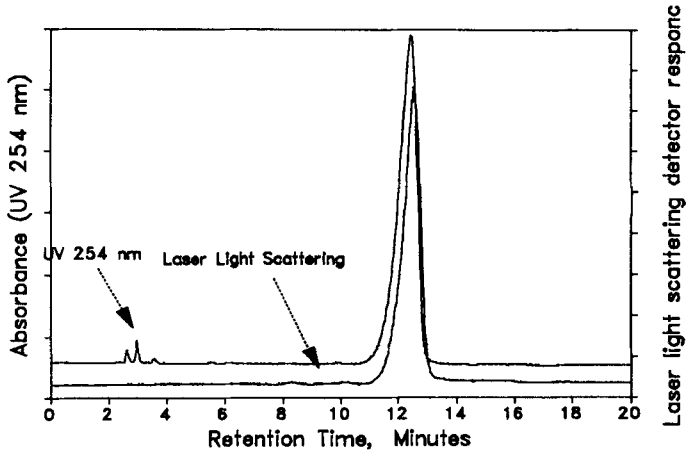

Figure 7. Analytical HPLC of AZT-MP-DG purified by preparative HPLC on an 2" A/E column.

evaporation, and AZT-MP-DG (1.2 gm) was obtained. Figure 7 shows the chromatogram of purified product on an analytical HPLC column. A UV 254 nm and a laser light scattering detector were used to analyze the purity of the product. The purified fraction was 99% pure by HPLC. The purity of the fractions was also tested by TLC.

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REFERENCES

1. Steim, J. M., Neto, C. C., Sarin, P. S. Sun, D., Sehgal, R. K. and Turcotte, J. G., *Biochemical and Biophysical Research Communications*, 171: 451 (1990)

2. Hostetler, K. Y., Stumiller, L. M., Lenting, H. B. M., van den Bosch, H. and Richman, D. M., *The Journal of Biological Chemistry*, 265: 6112 (1990)

3. Piantidosi, C., Marasco, C. J., Morris-Natschke, S. L., Meyer, F. G., Surles, J. R., Ishaq, K. S., Kucera, L. S., Iyer, N., Wallenc, A. and Piantidosi, S., *Journal of Medicinal Chemistry*, 34: 1408 (1991)

4. Steim, J. M., Neto, C. C., Buckheit, Jr, R. W., Shannon, W. M., Sehgal, R. K., Shirali, S. S., Singh, H. K., Wunz, T. W. and Turcotte, J. G., unpublished data.

5. Raetz, C. H. R., Chu M.-Y., Turcotte, J. G., *Science*, 196: 303 (1977)

6. Turcotte, J. G., Srivastava, S. P., Meresak, W. A., Rizkalla, B. H., Wunz, T. P., *Biochim. Biophys. Acta.*, 619: 604 (1980)

7. Turcotte, J. G., Srivastava, S. P, Steim, J. M., Calabresi, P., Tibbetts, L. M., Chu, M. Y., *Biochim. Biophys. Acta*, 619: 619 (1080)

8. Turcotte, J. G., "Cytotoxic Liponucleotide Analogs," U.S. Patent 4,291,024, (1981)

9. Yang, V. C. M., Turcotte, J. G., Steim, J. M., *Biochim. Biophys. Acta*, 689: 375 (1982)

10. Bermal, M. S., Turcotte, J. G., Steim, J. M., Notter, R. H., *J. Colloid Interface Sci.*, 115: 1647 (1987)

11. Van Wijk, G. M. T., Hostetler, K. Y., Schlame, M. and Bosch, V. D., *Biochimica et Biophysica Acta*, 99 (1991)

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