Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, Nos 10–12, pp. 871–875, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

High-performance liquid chromatographic analysis of 3′-azido-3′-deoxythymidine monophosphate diglyceride, an anti-HIV glycerophospholipid*

J.V. AMARI,† P.R. BROWN,†‡ P.E. PIVARNIK,§ R.K. SEHGAL§ and J.G. TURCOTTE‡§

† *Department of Chemistry and* § *Department of Medicinal Chemistry, University of Rhode Island, Kingston, RI 02881, USA*

Abstract: A reversed-phase chromatographic method is described for the analysis of an experimental anti-AIDS drug 3′-azido-3′-deoxythymidine monophosphate diglyceride (AZT-MP-DG) [J.M. Steim *et al.*, *Biochem. Biophys. Res. Commun.* **171**, 458–464 (1990)] [1], a phosphatidic acid derivative of AZT. Analytical conditions were based upon conventional separations of glycerophospholipid species. Where AZT-MP-DG was monitored by UV absorption, there were two wavelength maxima. The response was linear in the concentration range used in this study. The peak was characterized by absorbance ratios with a rapid scanning UV detector.

Keywords: *Reversed-phase chromatography; glycerophospholipid; liponucleotide; 3′-azido-3′-deoxythymidine monophosphate diglyceride (AZT-MP-DG).*

Introduction

Several stages in the replicative cycle [2, 3] of the human immunodeficiency virus (HIV) have been established or identified [4, 5] as molecular targets or potential sites for drug intervention. At present the only FDA-approved drug for the treatment of AIDS/ARC and early asymptomatic HIV infection [6] is the antiretroviral analogue of thymidine, 3′-azido-2′,3′-dideoxythymidine (azidothymidine, AZT) which inhibits reverse transcription (polymerase). AZT and other clinical dideoxynucleosides (e.g. ddI and ddC) exhibit dose-limiting toxicity [7, 8] and drug-resistant HIV are known. Further, clinical dideoxynucleosides exhibit less than ideal pharmacokinetics, as evidenced by short plasma half-lives of typically 30–60 min [8].

In an effort to decrease toxicity and increase efficacy, new experimental anti-AIDS drugs, stemming from earlier work on anti-cancer liponucleotides [9–12], have recently been developed [1] which are phosphatidic acid: nucleoside conjugates. One of these drugs is 3-azido-3′-deoxythymidine-5′-phosphate diglyceride (AZT-MP-DG) in which AZT is coupled to 16:0/18:1ω9 phosphatidic acid) (Fig. 1).

HPLC is a good techique for monitoring the synthesis of new pharmaceuticals and for assessing the purity of the final product. Therefore, we are reporting an analytical reversed-phase HPLC method for the analysis of AZT-MP-DG. This method, which was optimized for reproducibility, precision, linearity and resolution was used to determine the purity of AZT-MP-DG. The AZT-MP-DG

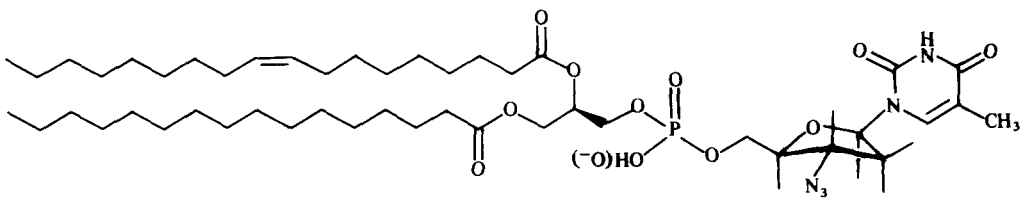

Figure 1
Structure of 3′-azido-3′-deoxythymidine monophosphate diglyceride (AZT-MP-DG).

* Presented at the "Third International Symposium on Pharmaceutical and Biomedical Analysis", April 1991, Boston, MA, USA.

‡ Authors to whom correspondence should be addressed.

was purified by two different preparative chromatographic methods [13].

Experimental

Reagents

All solvents (Fisher Scientific, Pittsburgh, PA) were of HPLC grade. The water, filtered through 0.45 μm Nylon-66 filters (AllTech. Assoc., Derrfield, IL), was doubly distilled and deionized. The mobile phase was degassed with He before use.

AZT-MP-DG

The semi-synthetic AZT-MP-DG [1] was obtained from the Department of Medicinal Chemistry, University of Rhode Island. The compound, a white powder, previously purified by open column adsorption (silica gel, 70–230 mesh) chromatography was considered to be an authentic standard. Purity was confirmed by TLC, NMR (^{1}H, ^{13}C, ^{31}P) and elemental analysis [1]. A 10 ml volume of a stock solution of 20 μg μl^{-1} was prepared. A mixture of $CHCl_3$–CH_3OH (8:2, v/v) was used as solvent and 10 μl of 0.01% butylated hydroxytoluene (BHT) in methanol was added as an antioxidant. To prepare a 2 μg μl^{-1} solution, a 2.5 ml aliquot of the stock solution was taken and diluted to 10 ml with $CHCl_3$–CH_3OH (2:1) containing 25 μl of the BHT solution. For the following standards the solvent consisted of $CHCl_3$–CH_3OH (1:2, v/v). A 1 μg μl^{-1} standard was prepared by diluting 5 ml of 2 μg μl^{-1} and 1 ml of the mobile phase to 10 ml with $CHCl_3$–CH_3OH (1:2). Serial dilutions were made with the 1 μg μl^{-1} solution for standards 1–4 in the following way: appropriate volumes of the 1 μg μl^{-1} solution were diluted with 1 ml of the mobile phase and brought up to the 10 ml mark with $CHCl_3$–CH_3OH (1:2). Subsequently, standards 5–7 were prepared in the same manner as 1–4, but with aliquots from the 0.1 μg μl^{-1} solution. To each standard, 10 μl of 0.01% BHT was added. The standards spanned a range from 0.005 to 0.1 μg μl^{-1}.

Another batch of AZT-MP-DG was synthesized by the same synthetic route but purified by the reversed-phase mode of HPLC.

Chromatography

The chromatographic system consisted of a Waters 6000 pump (Waters Chromatography Division, Millipore Corp., Milford, MA) a Rheodyne injector with 10 μl loop (Rheodyne, Berkeley, CA), a variable wavelength detector (Schoeffel Instrument Division, Kratos Inc. Westwood, NJ) and a rapid scanning UV detector (Barspec, Rehovot, Israel). For the linearity determination, the variable detector was used throughout at 267 nm at 0.02 AUFS The mobile phase, at a flow-rate of 1.5 ml min^{-1}, consisted of CH_3OH–1 mM potassium dihydrogen phosphate (KH_2PO_4) (95:5, v/v) at pH 2.4. All separations were performed isocratically at ambient temperature on a 5-μm, 25 × 0.40 cm, LiChrospher® 100 C_{18} column (EM Science, Gibbstown, NJ). All separations, except those monitored with the rapid scanning UV, were recorded on a HP 3393A integrator, 0.2 cm min^{-1} (Hewlett–Packard, Avondale, PA) and an OminiScribe strip chart recorder (Houston Instrument, Austin, TX).

Results and Discussion

Liponucleotides such as AZT-MP-DG are analogues of glycerophospholipids. Like naturally occurring glycerophospholipids, they have a polar head group and two non-polar fatty acyl chains esterified to the glycerol moiety. The physical properties of the liponucleotides are more similar to glycerophospholipids than nucleotides. Thus, separation conditions were based on phospholipid separations in which reversed-phase supports have been used with a mobile phase composed of 90–95% CH_3OH and 5–10% of 1–2 mM KH_2PO_4 [14, 15]. This solvent system, which permits detection in the low UV range (200–210 nm), is required, since phospholipids do not contain strong UV absorbing chromophores. However, detection methods for AZT-MP-DG can be based on the nucleotide moiety of the molecule, since the pyrimidine substituent absorbs strongly in the 250–280 nm region. Therefore, we were not limited in our choice of eluents to organic solvents that are transparent in the low UV region.

Methods development studies

Since the AZT-MP-DG purified by open column adsorption chromatography was not completely soluble in the mobile phase, chloroform was needed to dissolve it. In the three-dimensional chromatogram of AZT-MP-DG (Fig. 2) which was obtained with the rapid scanning UV, two absorbance maxima are

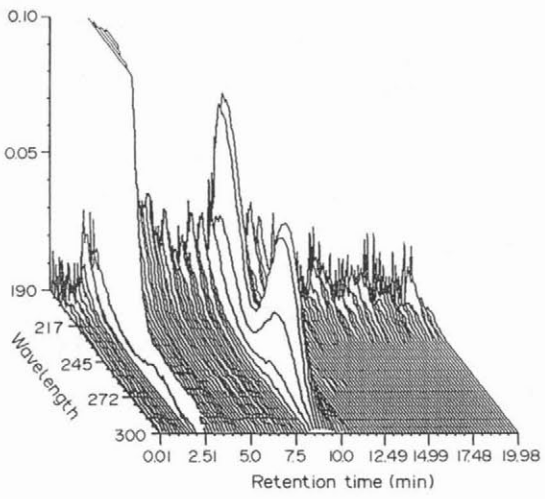

Figure 2
Three-dimensional chromatogram of 10 μg powdered AZT-MP-DG standard. Chromatographic conditions, mobile phase: 95:5 methanol–1 mM KH_2PO_4 (pH 2.4), flow-rate: 1.5 ml min^{-1}, column: LiChrospher® 100 C_{18}, detector: Barspec rapid scanning UV, scan rate: 10 spectra s^{-1}. Lambda maxima: 208 and 267 nm. Peak at 2.51 min is due to $CHCl_3$.

seen, one at 208 and the other at 267 nm. Either wavelength is suitable to monitor the separations. The wavelength maximum of 267 nm was used because of the strong absorption by the pyrimidine moiety at that wavelength. The UV spectrum of the AZT-MP-DG was identical to the nucleoside starting material, AZT, and the nucleoside intermediate, AZT-5′-MP. However, all three compounds have different retention times. The separation was also monitored at 220 and 208 nm for the purposes of peak characterization and to detect impurities that do not have a chromophore that absorbs in the 250–280 nm region of the UV. Therefore the low UV region must be used to detect these impurities. However, the $CHCl_3$ used to solubilize the AZT-MP-DG produced a large solvent peak at low wavelengths. Thus, impurities eluting near the solvent front may be obscured; on the other hand at 267 nm there is little absorption due to the $CHCl_3$. Peak splitting at all concentrations was observed when the AZT-MP-DG was dissolved in $CHCl_3$–CH_3OH due to the presence of the $CHCl_3$.

Another means of determining the purity of a compound is the ratio of absorbances at two wavelengths. Since reference values are not available for the AZT-MP-DG, the mean values of the three sets of ratios at (267/220, 267/208 and 220/208 nm) were 1.24, 0.855 and 0.690 with per cent RSDs of 3.40, 2.91 and 5.10, respectively ($n = 5$).

Purity determination. In order to detect trace contaminants in pharmaceutical analyses injections of high concentrations [16, 17] and the use of high sensitivity detectors [18] are required. For purity analysis, a high concentration of AZT-MP-DG was injected which produced a large peak at 267 nm. Minor components were detected, which eluted before and after the AZT-MP-DG. The impurities did not stem from the solvents used. The highest amount injected was 20 μg. The levels of impurities present were 0.24 and 0.57% for injections of 10 and 20 μg, respectively. The 0.57% was normalized with respect to the 10 μg injection, which resulted in a level of impurities of 0.29%. Thus the average per cent of impurities present was 0.26%. The contaminants have not been identified.

Reproducibility and precision. Various concentrations of AZT-MP-DG were injected to construct a calibration curve and determine the limit of detection. Each standard was injected five times, beginning with the lowest concentration. The calibration curve ranged from 55.0 to 1100.0 pmol. Representative chromatograms are shown in Fig. 3(a,b). Excellent reproducibility was obtained for retention times and the RSD ranged from 1.48 to 0.235. Constant temperature is required in order to obtain reproducible retention times as deviations were observed with fluctuations in temperature. Also, care must be taken when preparing the mobile phase; minor variations in either the organic or aqueous solvents can cause significant changes in retention times. Listed in Table 1 are the averaged retention times and areas for the standards. The relative standard deviation for standards of lower concentrations are larger than for those of higher concentration. The intercept and slope were −433.6 and 2176.9, respectively, with $R^2 > 0.9988$. The absolute error, 17658 ± 4959, is relatively constant throughout the working range. At approximately four times S/N, the limit of detection was 27 pmol.

AZT-MP-DG purified by reversed-phase HPLC

Fractions containing AZT-MP-DG were collected during a semi-preparative RP-HPLC separation [13]. The fractions containing the

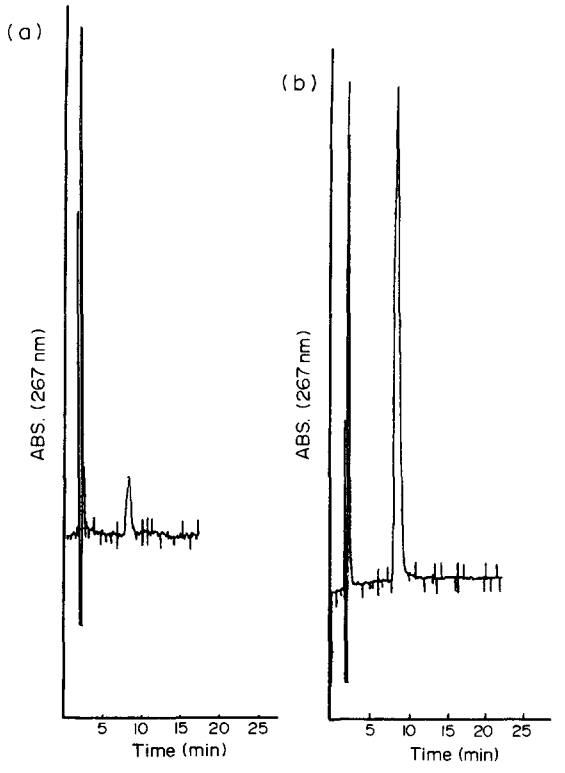

Figure 3
Representative chromatograms of AZT-MP-DG standards: (a) 55 pmole and (b) 1100 pmol. Detector: variable wavelength (267 nm), AUFS 0.02, chromatographic conditions same as in Fig. 2.

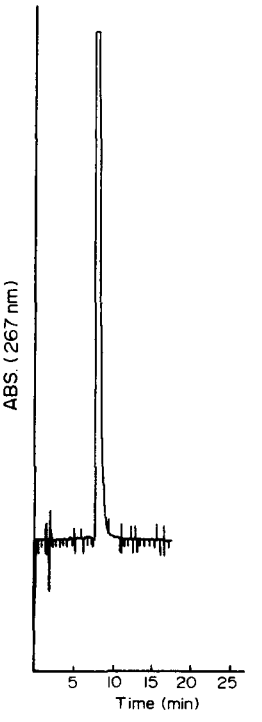

Figure 4
Analytical chromatogram of pooled AZT-MP-DG fractions isolated from preparative HPLC separation. Detector: variable wavelength (267 nm), AUFS 0.02, chromatographic conditions same as in Fig. 2.

AZT-MP-DG were not reduced to dryness and remained in the mobile phase used for the preparative separation. Therefore, chloroform was not present. A 10-μl aliquot of each collected fraction from the preparative separation was injected. The purest fractions were pooled, concentrated and analysed (Fig. 4). Trace impurities, 0.10%, were detected in the pooled fractions. Since each fraction was in the preparative mobile phase which consisted of CH_3OH–H_2O, it was possible to monitor the fractions at the low wavelengths without interference from the solvent peak.

Absorbance ratios at 267/220, 267/208 and 220/208 nm were determined for the pooled fractions. The mean values were consistent with those obtained previously and were 1.25, 0.893 and 0.713 with per cent RSDs of 2.88, 3.30 and 4.58, respectively (n = 3).

In summary, an RP-HPLC method for the analysis of a new experimental anti-AIDS drug, AZT-MP-DG, has been described. The purity of AZT-MP-DG for the powdered form was greater than 99.7%. The AZT-MP-DG isolated from the reversed-phase separation was greater than 99.8%. The compound was

Table 1
Calibration curve at 267 nm

AZT-MP-DG (pmol)	t_R Mean	t_R SD	t_R %RSD	Area Mean	Area SD	Area %RSD
55	8.29	0.12	1.48	118776	15971	13.4
82	8.59	0.071	0.83	200432	10510	5.24
110	8.82	0.046	0.53	269530	16338	6.06
275	8.69	0.047	0.54	575704	22652	3.93
550	8.65	0.074	0.85	1165700	13916	1.19
824	8.53	0.020	0.24	1719940	24685	1.43
1100	8.67	0.072	0.82	2462900	19537	0.79

Each value is the average of five injections.

characterized with a UV rapid scanning detector with which absorbance ratios were determined. Although commercial reference standards are not available, our results were consistent when our methods were used on two different batches of AZT-MP-DG. The AZT-MP-DG behaved in a linear manner. The analytical method described can be used to analyse other synthetic liponucleotides [13]. This reversed-phase method has been scaled to a preparative separation for the purification of liponucleotides [13].

Acknowledgements — The authors thank Barspec Ltd of Rehovot, Israel for the rapid scanning detector and Dr Fred Rabel of EM Science 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 of AIDS (NCDDG/HIV).

References

[1] J.M. Steim, C.C. Neto, P.S. Sarin, D.K. Sun, R.K. Sehgal and J.G. Turcotte, *Biochem. Biophys. Res. Commun.* **171**, 458–464 (1990).
[2] D.D. Ho, R.J. Pomerantz and J.C. Kaplan, *N. Engl. J. Med.* **317**, 278–286 (1987).
[3] W.C. Greene, *N. Eng. J. Med.* **324**, 308–317 (1991).
[4] H. Mitsuya, R. Yarchoan and S. Border, *Science* **249**, 1533–1544 (1990).
[5] E. DeClercq, *Trends Pharmacol. Sci.* **11**, 198–205 (1990).
[6] P.A. Volberding, S.W. Lagakos, M.A. Koch *et al.*, *N. Eng. J. Med.* **322**, 941–1004 (1990).
[7] D.D. Richman, M.A. Fiscl, M.H. Grieco, M.S. Gottlieb, P.A. Volberding, O.L. Laskin, J.M. Leedom, J.E. Groopman, D. Mildvan, M.S. Hirsch, G.C. Jackson, D.T. Durack, D. Phil, S. Nusinoff-Lehram and the AZT Collaborative Working Group, *N. Eng. J. Med.* **317**, 192–197 (1987).
[8] R. Yarchoan, H. Mitsuya, C.E. Myers and S. Border, *N. Eng. J. Med.* **321**, 726–738 (1989).
[9] C.H.R. Raetz, M.Y. Chu, S.P. Srivastava and J.G. Turcotte, *Science* **196**, 303–306 (1977).
[10] J.G. Turcotte, S.P. Srivastava, W.A. Meresak, B.H. Rizkalla and T.P. Wunz, *Biochem. Biophys. Acta* **619**, 604–607 (1980).
[11] J.G. Turcotte, S.P. Srivastava, J.M. Steim, P. Calabresi, L.M. Tibbetts and M.Y. Chu, *Biochem. Biophys. Acta* **619**, 619–623 (1980).
[12 V.C.M. Yang, J.G. Turcotte and J.M. Steim, *Biochem. Biophys. Acta* **689**, 375–379 (1982).
[13] J.V. Amari, P.R. Brown, P.E. Pivarnik, R.K. Sehgal and J.G. Turcotte, *J. Chromatogr.*, accepted.
[14] S.J. Robins and G.M. Patton, *J. Lipid Res.* **27**, 131–139 (1986).
[15] A. Cantafora, M. Cardelli and R. Masella, *J. Chromatogr.* **507**, 339–349 (1990).
[16] S. Ahuja, *J. Liq. Chromatogr.* **11**, 2175–2197 (1988).
[17] E.L. Inman and H.J. Tenbarge, *J. Chromatogr. Sci.* **26**, 89–94 (1988).
[18] M.W. Dong, P.V. Passalacqua and D.R. Choudhury, *J. Liq. Chromatogr.* **13**, 2135–2160 (1990).

[*Received for review 6 May 1991; revised manuscript received 27 August 1991*]