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PREPARATIVE-SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND PURIFICATION OF 3′-AZIDO-3′-DEOXYTHYMIDINE-5′-PHOSPHATE

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SUMMARY

Crude 3′-azido-3′-deoxythymidine-5′-phosphate (AZT-P), obtained from direct phosphorylation of 3′-azido-3′-deoxythymidine (azidothymidine, AZT), was separated and purified by isocratic preparative high-performance liquid chromatography. The components in a 2.5-g load of crude AZT-P, obtained from work-up of the phosphorylation reaction, were separated in 50 min to give 1.8 g of 99.5% pure AZT-P. AZT-P was analyzed by high-performance liquid chromatography and by high-resolution nuclear magnetic resonance (^{1}H, ^{13}C, ^{31}P) spectroscopy. The practical and rapid preparative chromatographic method is being applied to the purification of AZT-P and other antiretroviral dideoxynucleotides, used as intermediates in the synthesis of target-directed experimental drugs for the treatment of AIDS.

INTRODUCTION

3′-Azido-3′-deoxythymidine (azidothymidine, AZT), an antiretroviral analogue of thymidine, is presently the principal drug used for the clinical treatment of acquired immunodeficiency syndrome (AIDS) and early AIDS-related complex (ARC). It is used thereapeutically as a single agent or in combination with other anti-AIDS drugs. Since AZT exhibits hematologic toxicity and other undesirable side-effects, new target-directed derivatives of the drug are currently being synthesized[1]. For these syntheses, relatively large quantities of pure 3′-azido-3′-deoxythymidine-5′-phosphate (AZT-P) are required. Consequently, a reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the separation and purification of AZT-P on a preparative scale. Analytical HPLC methods have been reported (*e.g.* refs. 2–5) for the determination of the nucleoside AZT, its metabolically derived glucuronide, and other antiretroviral dideoxynucleosides in plasma, serum, and urine. However, little work is reported in the literature on the analysis of the nucleotide AZT-P, which is of interest in the monitoring of synthetic

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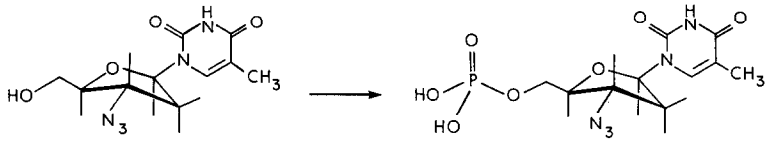
reactions in the purification of starting materials for the synthesis of AZT derivatives, and as an intracellular metabolite of AZT itself.



AZT AZT-P

EXPERIMENTAL

Materials

AZT was obtained from five different sources: by synthesis according to two independent routes[6–9]; as a gift from the U.S. National Cancer Institute; from commercial capsules of AZT (Zidovudine, Retrovir™); and from Sigma (St. Louis, MO, U.S.A.). AZT-P was synthesized by phosphorylation of AZT with phosphorus oxychloride–trimethylphosphate according to known procedures[9,10]. Potassium dihydrogenphosphate, ammonium acetate, and the solvents absolute methanol, chloroform, and diethyl ether (HPLC grade) were purchased from Fisher (Fairlawn, NJ, U.S.A.).

All solvents used in sample preparation and for the mobile phases were prepared with doubly distilled, deionized water, filtered through 0.45-μm Nylon-66 membrane filters (AllTech Assoc., Deerfield, IL, U.S.A.). Mobile phases, which were deaerated by helium, contained either KH_2PO_4 or ammonium acetate at a concentration of 10 m*M* and were mixtures of buffer and methanol. The pH of the mobile phase was adjusted to 5.0 with phosphoric acid or potassium hydroxide (Fisher). A standard solution of AZT, which was prepared by dissolving AZT in the buffer solution at pH 6.0, was stored at −20°C. A solution of the reaction mixture, which contained AZT-P and trimethylphosphate, was diluted with the solvent and kept at 4°C.

Since residual trimethylphosphate, the solvent used in the AZT phosphorylation reaction[9,10], was difficult to separate from the crude reaction mixture by distillation *in vacuo* at relatively low temperature (< 30°C), the following preliminary procedure was developed to remove the compound prior to preparative HPLC. Typically, crude AZT-P (5 g, containing trimethylphosphate) was dissolved in chloroform (18 ml, HPLC grade). A column of silica gel (190 g, mesh 70–230, "flash" type, EM Science, Cherry Hill, NJ, U.S.A.) was prepared in anhydrous diethyl ether, and the chloroform solution of crude AZT-P was loaded onto the column. It then was eluted sequentially with diethyl ether (600 ml), chloroform (700 ml), and chloroform–methanol (95:5, 300 ml). The solvent was drained from the column, the silicagel was emptied into a 1000-ml flask and stirred with chloroform–methanol (1:1) (3 × 250 ml), and then filtered through a sintered-glass funnel. The filtrate, upon evaporation of the solvent, gave a white solid (0.19 g, 0.21 g theoretical) which contained a slightly yellowish oily contaminant. The latter solidified upon cooling and addition of a small amount of hexane. Thin-layer chromatography (TLC); R_F 0.60, ammonium hydroxide–*n*-propanol–water (20:20:3, v/v/v).

Methods

The chromatograph used for analytical and methods development studies consisted of a Waters 6000A pump (Waters Division, Millipore, Milford, MA, U.S.A.), a Rheodyne 7125 injector (Rheodyne, Berkeley, CA, U.S.A.), and a Waters M440 absorbance detector at a fixed wavelength of 254 nm. The separations were performed on a Waters μBondapak C_{18} column (30 cm × 4.6 mm I.D., 10 μm particle size) with a guard column (5 cm × 4.6 mm I.D.) containing Whatman C_{18} pellicular packing (37–53-μm particle size). AZT and AZT-P were isocratically eluted with a mobile phase of 0.01 *M* KH_2PO_4–methanol (85:15, v/v), or 0.01 *M* ammonium acetate–methanol (85:15, v/v) (pH 5.0). The flow-rate was 1 ml/min. Data were recorded on both a HP 3390A integrator (Hewlet-Packard, Avondale, PA, U.S.A.) and an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). All separations were achieved at ambient temperature.

For analytical HPLC studies on the starting material itself, AZT, the conditions were: Perkin-Elmer (Norwalk, CT) C_{18} reversed-phase column (25 cm × 4.6 mm I.D., 10-μm particle size); mobile phase 0.01 *M* ammonium acetate–methanol (85:15, v/v; pH 5); flow-rate, 1 ml/min; sensitivity, 0.2 a.u.f.s.; chart-speed, 5 mm/min; detector, 254 nm; sample (AZT) size, 0.03 mg.

The methods development system was the same as the analytical system, except that the detector was a Knauer variable-wavelength detector (254 nm) (Sonntek, Woodcliff Lake, NJ, U.S.A.) and the column was a SepTech methods-development column (20 cm × 4.6 mm I.D., Separations Technology, Wakefield, RI, U.S.A.), containing C_{18} reversed-phase packing material (10–15-μm particle size, YMC, Newark, NJ, U.S.A.). Aliquots of the crude product were injected and eluted isocratically with a mobile phase of 0.01 *M* ammonium acetate–methanol (85:15, v/v; pH 5). Loading studies on larger amounts of crude product were also performed. The flow-rate was 1.0 ml/min.

A SepTech ST/800A preparative chromatograph (Separations Technology) with a SepTech Annular Expansion (A/E) C_{18} (YMC, 10–15-μm particle size) preparative column (20 cm × 7.3 cm I.D.), and a Knauer variable-wavelength detector (fixed wavelength, 254 nm) were used. A sample of 30 ml (equivalent to 2.5 g of crude AZT-5′-P) was injected into the system with a 10-ml syringe (Hamilton, Reno, NV, U.S.A.) flushed immediately with 10 ml of the mobile phase. AZT-P was eluted isocratically within 50 min with a mobile phase of 0.01 *M* ammonium acetate–methanol (85:15, v/v) at pH 5.0. The flow-rate was 150 ml/min. Data were recorded on both a strip-chart recorder and a HP 3393A integrator. Ten fractions were collected.

An aliquot of each preparative HPLC fraction was subsequently characterized on the analytical system. The fractions that contained the product were pooled, evaporated to a small volume, and lyophilized, giving a white, amorphous solid (AZT-P; diammonium salt); TLC, R_F 0.60, ammonium hydroxide–*n*-propanol–water (20:20:3, v/v/v); ^{1}NMR (300 MHz, ^2H_2O) δ 1.90 (singlet, 3H, methyl), 2.48 (triplet, 2H, H-2′), 4.06 (broad singlet, 2H, H-5′), 4.19 (broad singlet, 1H, H-4′), 4.50 (multiplet, 1H, H-3′), 6.27 (triplet, 1H, H-1′), 7.78 (singlet, 1H, H-6); ^{13}C NMR (75.4 MHz, ^2H_2O) δ 169.23 (C-4), 154.44 (C-2), 140.18 (C-6), 114.52 (C-5); 87.86 (C-1′), 86.10 (C-4′), 67.31 (C-5′), 63.60 (C-3′), 39.04 (C-2′), 14.33 (C-methyl); ^{31}P NMR (121.5 MHz, H_2O) δ 0.28 (broad singlet).

RESULTS AND DISCUSSION

AZT-P was synthesized in *ca.* 90% yield (crude) by direct phosphorylation (phosphorus oxychloride–trimethylphosphate)[10,11] from its dideoxynucleoside precursor, AZT. Analytical RP-HPLC of the starting material obtained by direct synthesis[10] and from commercial capsules (Retrovir) showed a broadening at the higher retention sides of the AZT peaks (retention time, 50 min) in each case, as well as a contaminant at a retention time of 5–6 min. That the contaminant is thymine was suggested by the retention time and by a chromatogram of a mixture with an authentic sample of thymine.

Optimal capacity factors (k') of AZT and AZT-P were obtained with a mobile phase of 0.01 *M* ammonium acetate–methanol (85:15, v/v) at pH 5.0. When the pH was 6.8 (the pH which has been used in HPLC assays for AZT has ranged from 2.2 to 7.1[2–5]), the AZT-P peak appeared as a triplet. This phenomenon has been observed in the HPLC of other nucleotides[11]. Therefore, a mobile phase, buffered to pH 5.0, was used for analytical, methods development, and preparative chromatography. AZT-P was eluted at 5.4 min and AZT at 13.8 min (Fig. 1); the k' values were 0.6 and 3.19, respectively. These k' values were adequate for the loading studies and the preparative separation. When the separation was scaled up to a methods-development column, good separation of AZT and AZT-P was obtained (Fig. 2). In a separate experiment, *ca.* 240 μl of crude reaction mixture were loaded on the column, and 24 mg of AZT-P were isolated. To facilitate removal of the mobile phase from the isolated AZT-P, a more volatile buffer, ammonium acetate, was used instead of KH_2PO_4. To

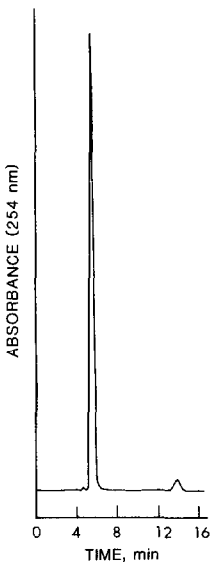


Fig. 1. Analytical RP-HPLC chromatogram of components in crude AZT phosphorylation reaction mixture. Conditions: Waters μBondapak C_{18} column (30 cm x 4.6 mm I.D., 10-μm particle size); mobile phase, 0.01 *M* KH_2PO_4–MeOH 5:15, v/v); pH 5.0; flow-rate, 1.0 ml/min; sensitivity, 0.05 a.u.f.s.; detector, Waters M440 absorbance detector; injection volume, 2 μl; sample size, 1.7 ng.

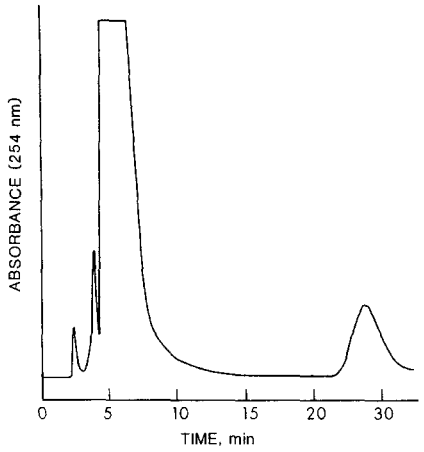


Fig. 2. Methods-development RP-HPLC chromatogram of separation of phosphorylation reaction mixture. Conditions: column, SepTech C_{18} (YMC) methods-development column (20 cm × 4.6 mm I.D., 10–15-μm particle size); mobile phase, 0.01 *M* ammonium acetate–methanol (85:15, v/v), pH 5.0; flow-rate, 1.0 ml/min; sensitivity, 2.0 au.f.s.; detector, Waters M440 absorbance detector (254 nm); injection volume, 100 μl; sample size, 10 mg).

prevent saturation of the detector a Knauer detector with a larger flow-cell was used instead of the Waters detector.

Impurities not seen in the analytical separation were observable when larger amounts of the crude reaction mixture were injected onto the column. These impurities were eluted prior to AZT-P. In the reaction mixture, unreacted AZT was also present. The scale-up factor for the preparative system was determined by the software from Separations Technology. A chromatogram of the crude product at a 2.5-g load (30 ml) is shown in Fig. 3. AZT, which has a longer retention time than AZT-P, was eluted at 40 min and did not interfere with recovery of the AZT-P. Ten fractions containing AZT-P were collected. These fractions were tested for the purity of the AZT-P with the analytical system; representative chromatograms are seen in Fig. 4. No AZT-P was recovered from fraction 1. Fraction 2 contained *ca.* 50% AZT-P and 50% impurities, including thymine. In fraction 3 the amount of impurities had de-

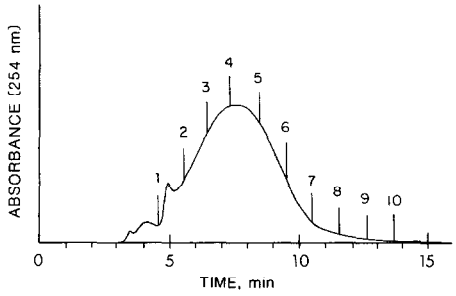


Fig. 3. Preparative RP-HPLC chromatogram showing the AZT-P and contaminants in the reaction mixture. Conditions: column, SepTech C_{18} (YMC) preparative column (20 cm × 7.3 cm I.D., 10–15-μm particle size); mobile phase, 0.01 *M* ammonium acetate–methanol 85:15, v/v); pH 5.0; flow-rate, 150 ml/min; detector, Knauer variable-wavelength (254 nm); injection volume, 30 ml; sample size, 2.5 g of crude reaction mixture. Numbers indicate fractions taken for analytical RP-HPLC (see Fig. 4).

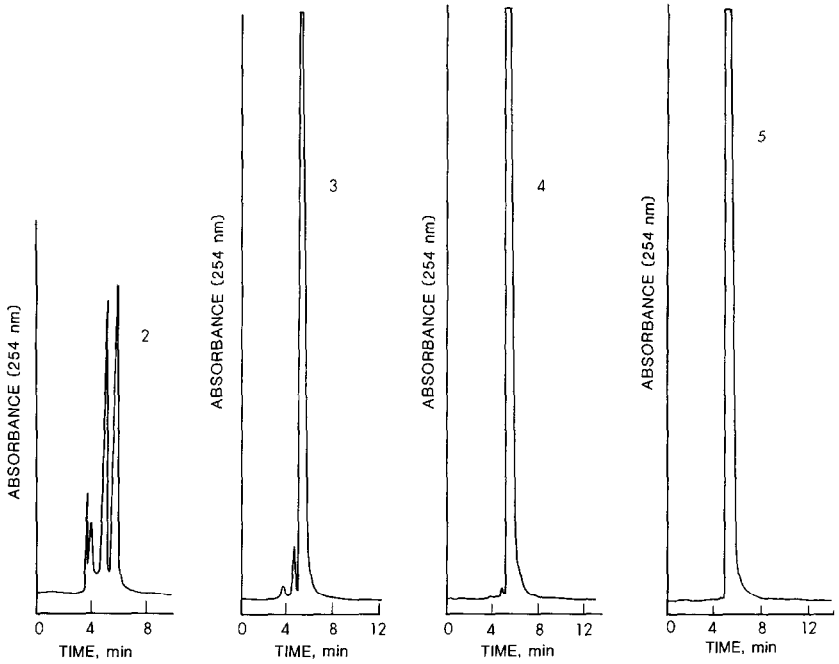


Fig. 4. Analytical RP-HPLC of aliquots of AZT-P-containing fractions (fractions 2–5) obtained from preparative RP-HPLC (Fig. 3). For chromatographic conditions, see Fig. 1.

creased to *ca.* 5%, and in fraction 4, only 0.7% of impurity was present. In fractions 5–10 the amount of impurity was negligible; relatively little AZT-P is present in fractions 8–10. In the pooled fractions 4–10, 1.81 g of AZT-P were obtained in 99.5% purity. When fractions 3–10 were pooled, 2.2 g of product (97.3% purity) were obtained. Four subsequent preparative HPLC runs gave comparable results.

The structure of the purified 3′-azido-3′-deoxythymidine-5′-phosphate was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy.

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