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Simultaneous analysis of azidothymidine and its mono-, di- and triphosphate derivatives in biological fluids, tissue and cultured cells by a rapid high-performance liquid chromatographic method

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ABSTRACT

A rapid high-performance liquid chromatographic (HPLC) method for the simultaneous analysis of the antiviral drug azidothymidine (AZT), AZT monophosphate, AZT diphosphate and AZT triphosphate, with ultraviolet detection in the nanomolar range, is described. Determination of these compounds *in vitro* in the human MT-4 lymphocyte cell line did not require a prior extraction, and AZT and its phosphorylated derivatives could be accurately analysed in one HPLC run. However, plasma, bile, liver homogenate and urine samples could not be injected directly into the chromatograph. Therefore, a solid-phase extraction procedure was developed, using azidodideoxyinosine as internal standard. The extractions of the compounds of interest from all but urine samples were reproducible, with recoveries between 65% (AZT triphosphate from plasma) and 100% (AZT from plasma).

INTRODUCTION

At present the most commonly used drug for the treatment of the acquired immune deficiency syndrome (AIDS) and AIDS-related symptoms is AZT (3′-azido-3′-deoxythymidine, Retrovir, Fig. 1). In 1985, Mitsuya *et al.* [1] reported that AZT is a very potent agent *in vitro* against human immunodeficiency virus type 1 (HIV-1). Although it has shown definite clinical effects in AIDS patients [2–4], AZT also induces toxic side-effects [2,5], the most severe being bone marrow depression [6].

Once inside the cell, the drug is phosphorylated to its active triphosphate form (AZTTP) [7], which interacts with the HIV-associated reverse transcriptase (RT). The triphosphate derivative of AZT acts either as a competitive inhibitor of RT or as an alternative substrate to thymidine of triphosphate, leading to integration into the newly formed DNA and subsequent inhibition of DNA chain elongation [8].

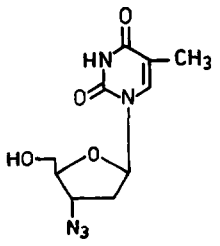

Fig. 1. Structure of 3′-azido-3′-deoxythymidine (AZT, Retrovir).

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The covalent attachment of AZT to a macromolecular carrier may result in a lower systemic concentration of the drug and may therefore reduce the toxic side-effects. This will also alter its pharmacokinetic behaviour *in vivo*, resulting in a diminished susceptibility to metabolic inactivation and clearance by the liver and the kidneys [5]. Also, by employing a carrier specifically recognized by the main target cells of HIV-1 (CD4-positive lymphocytes, monocytes and macrophages [9–16]), the selectivity of the drug can be increased.

Covalent binding of the 5′-monophosphate derivative of AZT (AZTMP) to some neoglycoproteins (human serum albumin chemically modified with various sugar derivatives) resulted in conjugates with potent effects against HIV-1 cytopathicity *in vitro* in the human T4 lymphocyte cell line MT-4 [17]. To unravel the mechanisms of the anti-HIV-1 activity of these conjugates, it is of great importance to learn more about the intracellular kinetics of the drug, administered to the cells in its free form and in its conjugated form.

The present study describes the development of a stable, rapid and sensitive high-performance liquid chromatographic (HPLC) method for the simultaneous analysis of AZT and its mono-, di- and triphosphate derivatives (AZTMP, AZTDP and AZTTP, respectively).

EXPERIMENTAL

Materials

Sodium dihydrogenphosphate, disodium hydrogenphosphate and tetrabutylammonium sulphate (TBAS) (Merck, Darmstadt, Germany) were of analytical grade. Acetonitrile (Labscan, Dublin, Ireland) was of HPLC grade. The water used was Milli-Q quality. AZT, AZTMP and AZTTP were kindly provided by Dr. P. Herdewijn, Rega Institute (Leuven, Belgium). 2′,3′-Dideoxyinosine (ddI), dideoxycytidine (ddC), 3′-azido-2′,3′-dideoxyinosine (azidoddI), 3′-azido-2′,3′-dideoxy-8-bromoadenosine (azidoddBrA) and 3′-fluoro-2′,3′-dideoxy-5-bromouridine (FddBrU) were kindly provided by Dr. J. Balzarini, Rega Institute. Alkaline phosphatase (type I-S, from bovine intestinal mucosa) was purchased from Sigma (St. Louis MO, USA). All other chemicals used were the best grade available.

Chromatographic method

The HPLC analyses were performed on a Waters liquid chromatograph (Waters, Milford, MA, USA) consisting of a 510 pump, a U6K injector and a Model 440 UV detector set at 254 nm mostly operating at 0.02 a.u.f.s. A Novapak C_{18} column, 15 cm × 3.9 mm I.D., 4 μm particle size (Waters), was used guarded with a μBondapak C_{18} Guard-pak precolumn (Waters). The eluent consisted of a 0.2 *M* sodium dihydrogenphosphate–disodium hydrogenphosphate buffer supplemented with 8 m*M* TBAS, set at pH 7.5, and acetonitrile. In method I the eluent was buffer–acetonitrile in a ratio of 95:5 (v/v) while in method II the ratio was 96.5:3.5. The flow-rate was 1.5 ml/min. Both methods are based on the method described by Ryll and Wagner [18], but adjusted based on the lipophilic character of the azido group present in the compounds of interest.

Reproducibility of the HPLC method

The day-to-day reproducibility of the HPLC method was determined by making a calibration curve of AZT, AZTMP and AZTTP on various days using freshly made buffer and eluent. Known amounts of the compounds were analysed using these daily calibration curves. Detection limits were defined as the concentrations at which the detector response was approximately equal to three times the detector noise.

Cell cultures

MT-4 cells were kindly provided by Dr. R. Pauwels, Rega Institute. The cells were grown in RPMI-1640 DM medium (Gibco, Life Technologies, Paisley, UK), supplemented with 10% (v/v) heat-inactivated foetal calf serum, 0.1% sodium bicarbonate, 2 m*M* glutamine (all purchased from Gibco) and 20 μg/ml gentamycin (Merck). Medium supplemented with the above-described ingredients will be referred to as "complete RPMI-1640 DM". The cells were maintained at

TABLE I

CHROMATOGRAPHIC FEATURES OF AZT, ITS PHOSPHORYLATED DERIVATIVES AND THE PROPOSED INTERNAL STANDARD COMPOUNDS USING METHOD I

The column used was Novapak C_{18}; the eluent consisted of 0.2 *M* phosphate buffer supplemented with 8 m*M* TBAS, pH 7.5, mixed with acetonitrile in a ratio of 95:5 (v/v). Flow was maintained at 1.5 ml/min. UV detection was set at 254 nm, 0.02 a.u.f.s. Values represent mean ± S.D. values of five experiments. Each experiment was performed on a different day, as described in the Experimental section.

Compound	Retention time (min)	Detection limit (n*M*)	Spiked amount (ng)	Calculated amount (ng)	Percentage of theoretical amount
AZT	6.5 ± 0.3	56 ± 6	136	141 ± 3	103
AZTMP	3.6 ± 0.1	90 ± 8	109	110 ± 6	101
AZTDP	5.3 ± 0.2	70 ± 10	N.D.[a]	N.D.	N.D.
AZTTP	8.2 ± 0.2	69 ± 6	168	169 ± 3	101
ddI	1.4 ± 0.1	107 ± 13	N.D.	N.D.	N.D.
ddC	1.1 ± 0.1	120 ± 15	N.D.	N.D.	N.D.
AzidoddI	4.0 ± 0.2	36 ± 4	200	190 ± 8	95
AzidoddBrA	>30	N.D.	N.D.	N.D.	N.D.
FddBrU	8[b]	N.D.	N.D.	N.D.	N.D.

[a] N.D. = not determined.

[b] The peak of FddBrU displayed a large degree of tailing.

37°C in a humidified atmosphere of 5% carbon dioxide in air. Every three or four days, the cell suspension was homogenized and seeded at $3 \cdot 10^5$ cells/ml.

Incubation experiments

The intracellular amounts of AZT and its derivatives were determined according to the following protocol: MT-4 cells were split 24–48 h before use at $3 \cdot 10^5$ cells/ml in complete RPMI-1640 DM, achieving cells in exponential growth at the beginning of the incubation. Exact determination of the cell concentration and viability (using Trypan Blue exclusion) was performed just before the incubation started. Cells were incubated with known concentrations of AZTMP using the conditions described above in the *Cell cultures* section. After 6 h, 6 ml of the cell suspensions were pelleted at 380 *g* for 5 min at 4°C and the supernatant was discarded. Cell pellets were washed twice with 1 ml of cold phosphate-buffered saline (PBS) and centrifuged at 380 *g* for 5 min at 4°C. Cell lysis took place by resuspending the pellet in 200 μl of PBS and freeze–thawing five times, using liquid nitrogen. Pelleting of cell debris was performed at 3200 *g*, 4°C, for 10 min. A 20- to 50-μl aliquot of the supernatant was analysed by HPLC.

Alkaline phosphatase incubation

Cell lysis supernatant was incubated with alkaline phosphatase to confirm that the peaks detected in the various chromatograms were AZT metabolites: a decrease in the peak height of the metabolite accompanied by an increase in the AZT peak height upon incubation with this enzyme strongly indicates that the peak under investigation is an AZT metabolite. Alkaline phosphatase at 0.75 mg/ml was dissolved in a 0.2 *M* Tris–1 m*M* magnesium chloride buffer pH 9.5. A 200-μl aliquot of this alkaline phosphatase solution was mixed with 100 μl of cell lysis supernatant. The mixture was incubated for 1 h at 37°C and analysed. AZT, AZTMP and AZTTP were incubated in the same way as a control experiment.

Internal standard

Based on knowledge of the lipophilicity of the nucleosides and nucleotides analysed by methods

I and II, the following compounds were proposed for internal standard use: ddI, ddC, azidoddI, azidoddBrA and FddBrU. The compounds were dissolved at 1 mg/ml in distilled water and analysed using method I. The results are shown in Table I. Based on these results, we chose azidoddI as internal standard for the extraction.

Extraction procedure

For the determination of AZT and its phosphorylated derivatives in blood samples, urine, bile and tissue homogenates, an extraction procedure on an SPE system (Baker, Phillipsburg, NJ, USA) was developed.

To water, plasma (human, cat, rat), bile (rat), urine (human, rat) and liver homogenate (rat), known amounts of the compounds of interest and azidoddI as internal standard were added. The C_{18} disposable extraction columns were prepared by treatment with successive solvent washes of 3 × 1 ml of methanol, 1 ml of distilled water and 3 × 1 ml 0.2 *M* sodium phosphate buffer supplemented with 8 m*M* TBAS, pH 7.5. Aliquots of 200 μl of the biological samples were diluted with 200 μl of buffer and loaded on the SPE column. After washing with 2 × 200 μl of buffer, the SPE column was allowed to dry. Elution took place with 2 × 100 μl of methanol and the combined methanol eluates were mixed with an equal amount of distilled water to prevent the formation of precipitate. A 40-μl aliquot of the mixture was injected into the chromatograph.

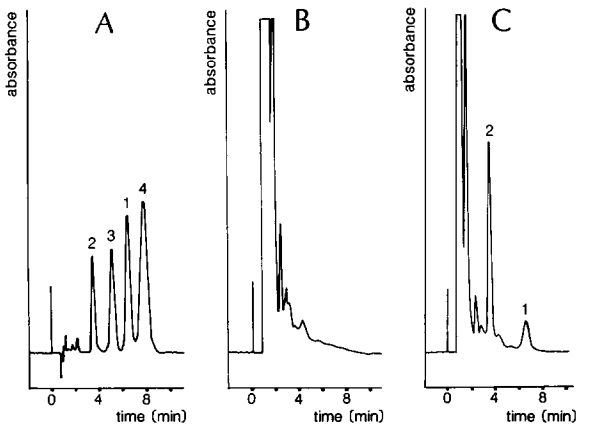

Fig. 2. (A) Typical chromatogram of a mixture of AZT and its mono-, di- and triphosphate derivatives in distilled water. (B) Chromatogram of MT-4 cell lysis supernatant. (C) Chromatogram of MT-4 cell lysis supernatant after incubation with AZTMP, analysed using method I. Peaks: 1 = AZT; 2 = AZTMP; 3 = AZTDP; 4 = AZTTP. A more detailed description of the method is given in the Experimental section.

RESULTS AND DISCUSSION

Figs. 2 and 3 show typical chromatograms of AZT and its mono, di- and triphosphate derivatives using methods I and II, respectively. The use of buffer–acetonitrile at a ratio of 95:5 (v/v) resulted in a separation of the four compounds within 10 min. Lowering the concentration of acetonitrile to 3.5% gave rise to a better separation between the solvent front and the first-eluting nucleotide (AZTMP), which may be an advantage in case of early-eluting contaminants (Fig. 3A). However, running time was prolonged to about 22 min.

Tables I and II show the results of the day-to-day reproducibility of both methods. Noteworthy is the change in the eluting sequence of AZT and AZTDP between methods I and II: retention times of AZT and AZTDP were 6.5 ± 0.3 and 5.3 ± 0.2 min with method I and 11.3 ± 1.0 and 13.0 ± 1.0 min with method II (mean ± S.D., n = 5). From these tables it can be concluded that both methods are reliable and give reproducible results over a period of time. Linear calibration curves for the compounds can be obtained in the nanomolar range [correlation coefficients varied between 0.99822 ± 0.00101 and 0.99924 ± 0.00065 (mean ± S.D., n = 5)]. We commonly observed some variation in the slope of the calibration curves from day to day. However, this only implies the need for a daily calibration curve. As can be seen from Tables I and II, daily determination of known amounts of the compounds can be carried out accurately. In the case of method II it should be stressed that eluting the column overnight with methanol is essential to maintain the (longer) retention times and separation as in Fig. 3A. Omitting this re-equilibration step will lead to slowly decreasing retention times of all compounds and non-reproducible results during the analysis.

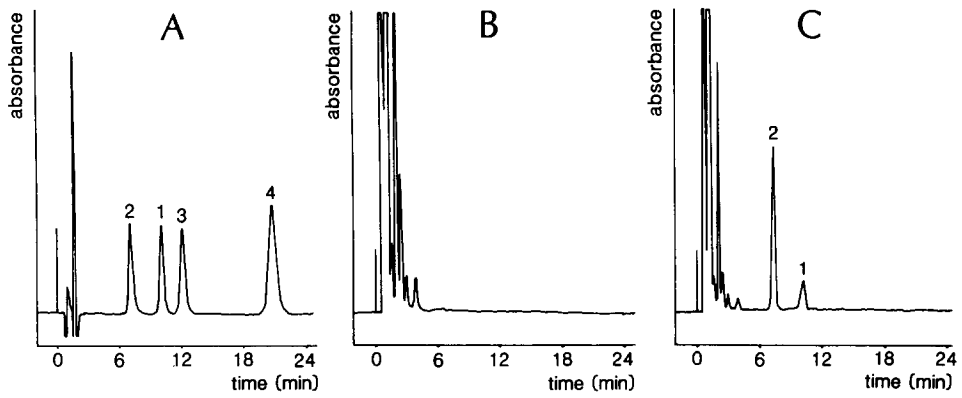

Fig. 3. (A) Typical chromatogram of a mixture of AZT and its mono-, di- and triphosphate derivatives in distilled water. (B) Chromatogram of MT-4 cell lysis supernatant. (C) Chromatogram of MT-4 cell lysis supernatant after incubation with AZTMP, analysed using method II. Peaks: 1 = AZT; 2 = AZTMP; 3 = AZTDP; 4 = AZTTP. A more detailed description of the method is given in the Experimental section.

Incubation of MT-4 cells with AZTMP resulted in an intracellular amount of AZTMP that was dependent on the extracellular concentration (Fig. 4). Very small amounts of AZT could be detected (less than 10 ng per 10^6 cells). Within 2 h of incubation in the complete RPMI-1640 DM medium, AZTMP was completely hydrolysed to AZT. AZT apparently diffused passively into the cells and was phosphorylated to AZTMP by the cytosol thymidine kinase [7,19,20]. Accumulation of AZTMP, as can be seen from Fig. 4, indicates that the thymidylate kinase is the rate-limiting step in the anabolism of AZT to AZTTP, as has been shown for other cell types [7]. The identity of the peak at retention time of 3.6 min was established using incubation with alkaline phosphatase (Fig. 5).

The finding that no interference by endogenous compounds occurred can be explained by the fact that all endogenous nucleosides and nucleotides possess a 3′-hydroxyl group, whereas AZT and its derivatives have a 3′-azido substituent (see Fig. 1). This makes the latter compounds far more lipophilic (endogenous compounds, *e.g.* adenosine, thymidine, cytidine, guanosine and uridine, were not retained at all in the methods used). Even the antiviral compounds ddI and ddC (compounds lacking the hydrophilic 3′-OH moiety) eluted within 2 min.

TABLE II

CHROMATOGRAPHIC FEATURES OF AZT AND ITS PHOSPHORYLATED DERIVATIVES USING METHOD II

The column used was Novapak C_{18}; the eluent consisted of 0.2 *M* phosphate buffer supplemented with 8 m*M* TBAS, pH 7.5, mixed with acetonitrile in a ratio of 96.5:3.5 (v/v). Flow was maintained at 1.5 ml/min. UV detection was set at 254 nm, 0.02 a.u.f.s. Values represent mean ± S.D. values of five experiments. Each experiment was performed on a different day, as described in the Experimental section.

Compound	Retention time (min)	Detection limit (n*M*)	Spiked amount (ng)	Calculated amount (ng)	Percentage of theoretical amount
AZT	11.3 ± 1.0	150 ± 8	242	254 ± 1	105
AZTMP	7.6 ± 0.5	140 ± 10	223	216 ± 8	97
AZTDP	13.0 ± 1.0	115 ± 7	N.D.[a]	N.D.	N.D.
AZTTP	22.5 ± 1.9	200 ± 15	179	185 ± 9	103

[a] N.D. = not determined.

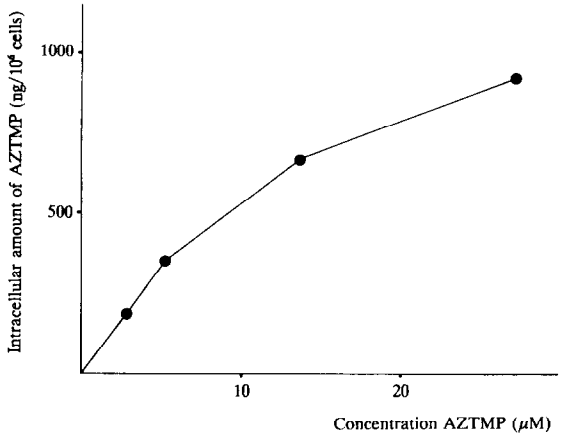

Fig. 4. Intracellular accumulation of AZTMP after 6 h incubation of MT-4 cells under the conditions described in the Experimental section with increasing concentrations of AZTMP.

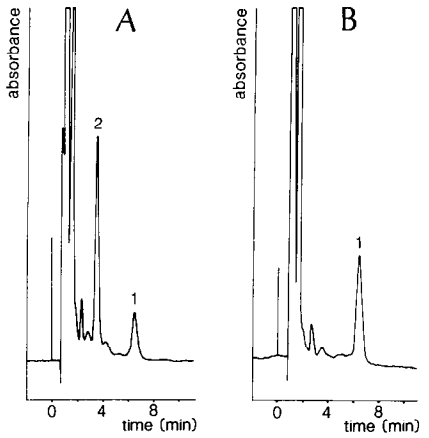

Fig. 5. Identification of AZTMP, present in MT-4 cells after incubation with AZTMP, using alkaline phosphatase (1 = AZT; 2 = AZTMP). (A) Chromatographic pattern of the cell lysis supernatant of MT-4 cells. (B) Chromatographic pattern after incubation of the cell lysis supernatant with alkaline phosphatase. (The HPLC method used is method I.)

Intracellular AZTDP and AZTTP could not be detected with the present method owing to the extremely low levels achieved in the cells, as shown by others [20–22]. Although each sample contained $3 \cdot 10^6$–$6 \cdot 10^6$ cells, the total amounts of AZTDP and AZTTP remained below the UV absorbance detection limits. Performing the experiments with radiolabelled AZT could in principle lower this detection limit considerably [7,20,22].

Whereas the MT-4 cell lysis supernatants presented a relative clean chromatographic pattern, in the other biological samples, such as tissue homogenates, plasma and bile, AZT and its derivatives could only be studied after extraction. Using method I, of the compounds tested for internal standard use, FddBrU and azidoddBrA had retention times of 8 min and >30 min, respectively (Table I). Furthermore, the peak of FddBrU was broad and showed a high degree of tailing. These characteristics made neither of these compounds suitable for internal standard use. However, the compound azidoddI showed an appropriate retention time and good peak shape and extraction characteristics. Table III shows the recoveries of AZT and its derivatives from the biological samples. The percentages recoveries were related to that of azidoddI, which was more than 95% from all but the urine samples. AZT showed a good and very reproducible recovery from all biological fluids as well as from distilled water. Although the recoveries of AZTMP were slightly less in the biological samples, they were highly reproducible. AZTTP could be extracted in its original triphosphate form only from distilled water. It was unstable in plasma, tissue homogenate and bile. Therefore, the recovery values were the sum of recovered AZTMP, AZTDP and AZTTP. Furthermore, it was impossible to extract the compounds from (human and rat) urine, owing to the interference of several endogenous compounds in the chromatograms.

Experiments are now in progress to study the behaviour of some of the AZTMP–neoglycoprotein conjugates in MT-4 cells with regard to the mechanism(s) underlying the anti-HIV-1 activity, using the methods described above.

In conclusion it can be said, that, compared with the analyses published so far (*e.g.* refs. 7, 20, 21, 23 and 24), the HPLC methods described above have three major advantages. First, AZT and its phosphorylated derivatives can be well separated within 10 min; second, the methods described utilize relatively simple isocratic elution; and third, the compounds can be detected in the

TABLE III

RECOVERIES OF AZT, AZTMP AND AZTTP FROM VARIOUS BIOLOGICAL SAMPLES, USING THE "BAKER"-10 SPE SYSTEM

SPE columns were successively pretreated with methanol, distilled water and 0.2 *M* phosphate buffer supplemented with 8 m*M* TBAS, pH 7.5. After loading and washing the samples, compounds of interest were eluted with 200 μl of methanol. The methanol was diluted 1:1 with distilled water and 40 μl were injected into the chromatograph. The recovered amounts are compared with the internal standard azidoddI. Values represent mean ± S.D. values of five experiments.

Biological sample	Recovery (%)		
	AZT	AZTMP	AZTTP
200 μl of water	104.2 ± 3.9	100.1 ± 2.7	79.1 ± 3.1
200 μl of plasma	106.0 ± 5.4	83.6 ± 3.2	65.3 ± 9.1[a]
200 μ of liver homogenate	100.9 ± 1.3	77.8 ± 2.9	71.6 ± 8.4[a]
100 μl of bile	95.5 ± 4.1	87.4 ± 2.2	73.3 ± 8.7[a]
100 μl of urine	—	—	—

[a] AZTTP was unstable in these biological samples. Because of this, the recovery was inferred from the recoveries of AZTMP, AZTDP and AZTTP.

nanomolar range using UV detection. Furthermore, extraction of AZT and its metabolites from bile, plasma and tissue homogenates can be easily and reproducibly performed using a solid phase method.

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