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Quantification of zidovudine and its monophosphate in cell extracts by on-line solid-phase extraction coupled to liquid chromatography

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Abstract

A simple and rapid analytical method for the simultaneous quantification of zidovudine (AZT) and its monophosphate (AZTMP) in cell extracts has been developed using high-performance liquid chromatography (HPLC) with on-line solid-phase extraction and 2-aminoethyl-3′-azido-2′,3′-dideoxythymidin-5′-yl phosphodiester sodium salt as internal standard (IS). The cell extract samples were directly injected on a short reversed-phase precolumn using an aqueous buffer containing an ion-pairing reagent as a mobile phase. Under these conditions, the analytes were retained on the precolumn whereas the proteins were discarded. The analytes were then transferred onto the analytical column by increasing the strength of the eluent. The calibration curve was linear over a concentration range of 0.5–100 μg/ml. Inter- and intra-day accuracy and precision results satisfied the accepted criteria for bioanalytical validation. This method was used to study the decomposition pathway of a model pronucleotide in an *in vitro* approach.

Keywords: Zidovudine; Zidovudine monophosphate; On-line solid-phase extraction; Ion pair; Cell extracts

1. Introduction

Zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) is a nucleoside analog with activity against the human immunodeficiency virus (HIV). It was the first anti-HIV agent to be approved by the FDA for the treatment of AIDS [1]. As with other chemotherapeutic nucleoside analogs [2], the antiretroviral effect of AZT involves its conversion into the corresponding 5′-mono-, di-, and triphosphates by cellular kinases. AZT triphosphate (AZT-TP) then competes with endogenous nucleotide, deoxythymidine triphosphate (dT-TP) for incorporation in viral DNA, leading to inhibition of reverse transcriptase and DNA chain termination. This dependence on kinase-mediated phosphorylation may limit the efficiency of AZT in a cellular environment where nucleoside kinase activity is low, as in macrophages [3] or in cells that have lost their ability to phosphorylate AZT after prolonged exposure to the drug [4,5]. In order to circumvent this problem and deliver the monophosphate efficiently into the target cell, prodrug strategies [6] were developed. The objective of the present study was to develop and to valid a simple HPLC method to quantify AZT and AZTMP (Fig. 1), metabolites formed after incubation of prodrugs in cell extracts. This biological medium was used to mimic the behaviour of the pronucleotides inside the targeted cells.

Some HPLC methods for quantifying AZT [7–9] or AZTMP [10] in biological media have been published, but only a few are able to determine AZT and AZTMP simultaneously [11–14]. To isolate AZT and/or AZTMP from other endogenous compounds and remove macromolecular compounds such as proteins, these methods require tedious preliminary extraction and cleaning stages of the sample based mainly on solid-phase extraction (SPE) and liquid–liquid extraction. To avoid sample pretreatment prior analysis, direct injection of biological samples involving the use of precolumns before the analytical column and back-flushing techniques were developed [15]. In

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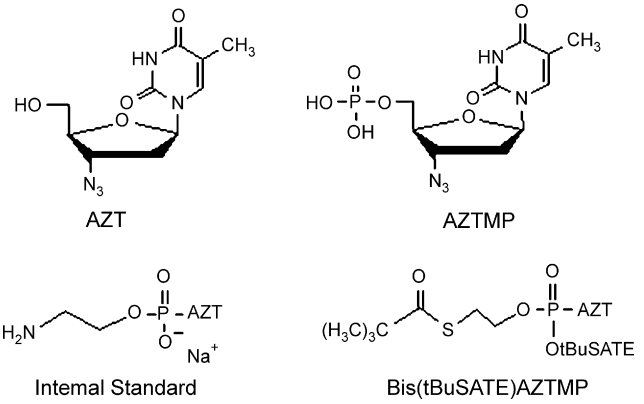

Fig. 1. Chemical structures of AZT, AZTMP, internal standard and studied pronucleotide.

this paper, an on-line solid-phase extraction coupled to LC/UV detection method to simultaneously quantify AZT and AZTMP in cell extracts is reported. Method development, validation of the analytical procedure and its application to assess the degradation kinetics of an AZT pronucleotide in cell extracts are discussed.

2. Experimental

2.1. Chemicals and reagents

Zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) was purchased from Sigma (St. Louis, MO, USA) and 2-aminoethyl-3′-azido-2′,3′-dideoxythymidin-5′-yl phosphodiester sodium salt, used as internal standard (IS) was synthesized in our laboratory (unpublished data). The synthesis of 3′-azido-2′,3′-dideoxythymidine-5′-monophosphate (AZTMP) was carried out following a general procedure [16]. The chemical purity of the compounds was greater than 99%. Total cell extracts from CEM-SS cells were kindly prepared by Dr. A.-M. Aubertin (Université Strasbourg I, France) according to the following procedure: CEM cells in logarithmic growth were separated from their culture medium by centrifugation ($10^4 \times g$, 4 min, 4 °C). The residue was resuspended in 2 ml of buffer (Tris–HCl 10 mM, KCl 140 mM, pH 7.4) and sonicated. The lysate was centrifuged ($10^5 \times g$, 1 h, 4 °C) to remove membranes, organelles and chromatin. The upper layer was filtered (0.22 μM, Millex GV) and stored in a sterile container at −20 °C. Phosphatase inhibitor cocktails 1 and 2 were purchased from Sigma (St. Louis, MO, USA). Phosphatase inhibitor cocktail 1 was described to inhibit the L-isozymes of alkaline phosphatases as well as serine/threonine protein phosphatases and phosphatase inhibitor cocktail 2 was described to inhibit acid and alkaline phosphatases as well as tyrosine protein phosphatases.

Deionised water purified by a Millipore water purification system (Millipore Corporation, Bedford, MA, USA) was used in all sample preparation and analysis. Acetonitrile (Fisons, Loughborough, UK) was of HPLC grade. Triethyl ammonium acetate buffer (2 M, pH 6.6) was purchased from Distribio (France). All solvents and buffers were filtered through 0.45 μm GV-type membrane (Millipore) before use.

2.2. Instruments

Chromatographic analysis were made on an Alliance 2690 system (Waters, Milford, MS) equipped with a Model 996 photodiode array UV-absorbance detector, an auto-sampler and a Millenium data workstation controlling the two automated six-port 7010 Rheodyne switching valves (Phase Separation, Pessac, France).

2.3. Analytical procedures

Without any other pretreatment, crude aliquot was directly injected on a cleaning precolumn (Waters, Guard-Pak, Inserts Delta-Pak C_{18}, 5 μm). Using an ion-pairing agent (eluent A: 20 mM triethylammonium acetate buffer, pH 6.6), AZT, AZTMP and IS were trapped on the precolumn while proteins and other unwanted compounds were quickly eluted to waste (Fig. 2). Then, the precolumn was connected to a reversed-phase analytical column (Waters, Symmetry C_{18}, 50 mm × 2.1 mm, 3.5 μm). The analytes were eluted using a linear gradient from 100% eluent A to 35% eluent B (eluent A containing 80% acetonitrile) over 20.0 min. Analytes were detected with UV at 266 nm. The flow rate was 0.3 ml/min and the temperature was 30 °C.

2.4. Preparation of standard solutions

Stock solutions of AZT, AZTMP and IS were prepared in water at a concentration of 4 mg/ml and stored at −20 °C. These solutions were further diluted with water to obtain standard solutions of AZT and AZTMP at a concentration of 200 μg/ml.

2.5. Preparation of calibration standards

Cell extracts samples containing phosphatase inhibitor were spiked with standard solutions to obtain calibration standards of 0.5, 1, 5, 10, 25, 50, 75, 100 for AZT and AZTMP and 50 μg/ml for IS. The spiked cell extract samples were prepared just before analysis.

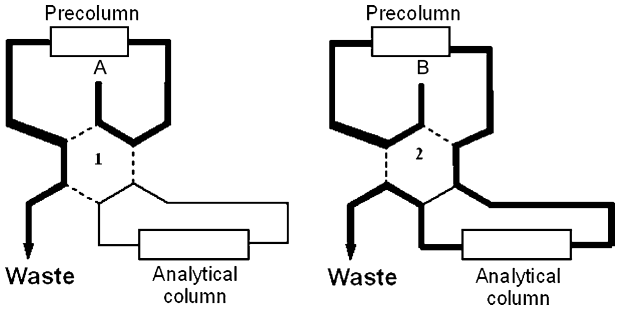

Fig. 2. The on-line cleaning concept for direct HPLC analysis of biological samples.

2.6. Method validation

2.6.1. Stability in cell extracts

For each kinetic study, 100 μl of a stock solution of AZTMP (AZT or IS) 10^{-3} M was diluted to 2 ml with cell extracts or in cell extracts containing phosphatase inhibitor cocktail 1 or 2 (20 μl) to obtain an initial concentration of 31 μg/ml. The resulting solutions were vortex-mixed then divided among HPLC autosampler inserts. Sterile procedures were used throughout. For each assay, the inserts were incubated at room temperature for the required time and immediately frozen at −80 °C.

2.6.2. Specificity

Six different sources of blank cell extracts were analyzed to study potential interferences from endogenous substances with AZT, AZTMP or IS.

2.6.3. Recovery

The recoveries for AZT and AZTMP were evaluated by comparing the peak area ratios (Analyte/IS) of cell extract samples with those from non-extracted standard solutions prepared in water at the same concentrations. Recovery was evaluated at levels of 0.5, 50 and 100 μg/ml ($n = 6$ at each level).

2.6.4. Lower limit of quantification (LLOQ)

The LLOQ for AZT and AZTMP was determined using five independently prepared cell extract samples at a concentration level of 0.5 μg/ml. The LOD experimentally determined as the concentration of AZT and AZTMP giving a signal/noise ratio equal to 3.

2.6.5. Linearity

The linearity of the calibration curve for AZT and AZTMP was studied by analyzing eight calibration standards in the range 0.5–100 μg/ml in triplicate, independently. The calibration curves were studied by plotting the peak area ratios (AZT/IS or AZTMP/IS) versus the concentration added (AZT or AZTMP).

2.6.6. Accuracy and precision

Accuracy and precision were evaluated by analyzing cell extract samples spiked with AZT or AZTMP at 0.5, 50 and 100 μg/ml from six measurements conducted within a day (intra-day precision) and from three measurements conducted on three separate days (inter-day precision). These levels were chosen to demonstrate the accuracy and precision of the method at low (LLOQ), medium and high level of concentration.

3. Results/discussion

The purpose of this study was to develop an HPLC analytical method for the simultaneous determination of AZT and AZTMP in cell extracts. AZTMP is progressively dephosphorylated to AZT by phosphatases present in this medium. The proposed analytical method does not require any sample pretreatment prior injection.

3.1. Method development

2.5 μl of AZT (4 mg/ml), 2.5 μl of AZTMP (4 mg/ml) and 2.5 μl of IS (4 mg/ml) were mixed in a tube with 200 μl of cell extracts containing phosphatase inhibitor cocktail 2 or with 200 μl of water, and vortexed briefly. 80 μl of this crude sample (initial concentration 50 μg/ml) were injected onto the precolumn and first analyzed with eluent A at a flow rate of 2 ml/min for 2.5 min (Fig. 2). Then, by activating the six-port Rheodyne valve and back-flushing the precolumn with a linear gradient from 100% eluent A to 35% eluent B for 20 min at a flow rate of 0.3 ml/min, the retained analytes were transferred and concentrated to the front of the analytical column where they were separated and quantified. Under these chromatographic conditions, AZTMP eluted at 11.3 min, IS at 12.6 min and AZT at 15.0 min.

Concerning the nature of the precolumn, various commercial stationary phases were tested. When using restricted access material (RAM) such as LiChrospher (RP-18 ADS) precolumn (25 mm × 4 mm, 5 μm, Merck), AZT was retained on the precolumn but AZTMP was eluted near the solvent front with proteins from the biological medium. Various reversed-phase packing materials (Resolve, Delta-Pak, Nova-Pak, all from Waters) were tested. The Delta-Pak material lead to better elimination of endogenous compounds from cell extracts during the isolation stage and was therefore selected for further method development.

Reversed phase is the most widely employed technique in pharmaceutical analysis to study small drug molecules which are separated by their degree of hydrophobic interaction with the stationary phase. However, for the determination of drugs with low octanol–water partition coefficients (log P), it is very challenging to establish a chromatographic method because these polar

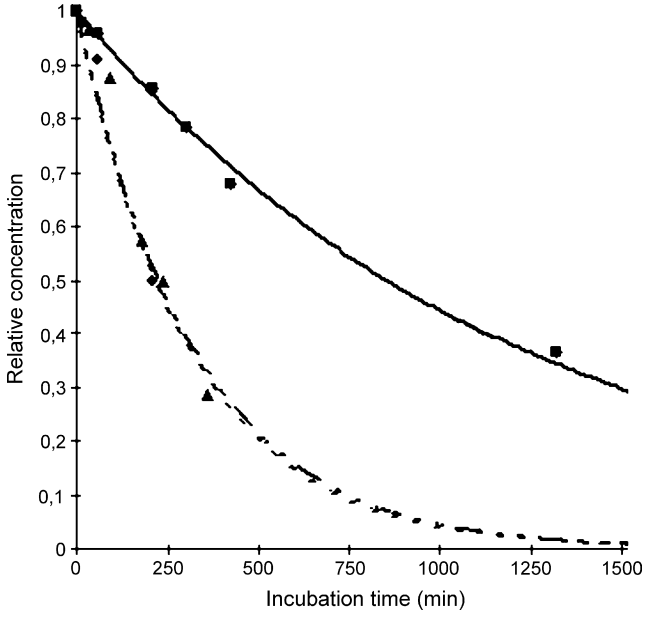

Fig. 3. Decomposition kinetics of AZTMP at 37 °C in total CEM-SS cell extracts (▲) with phosphatases inhibitor cocktail 1 (●) and 2 (■).

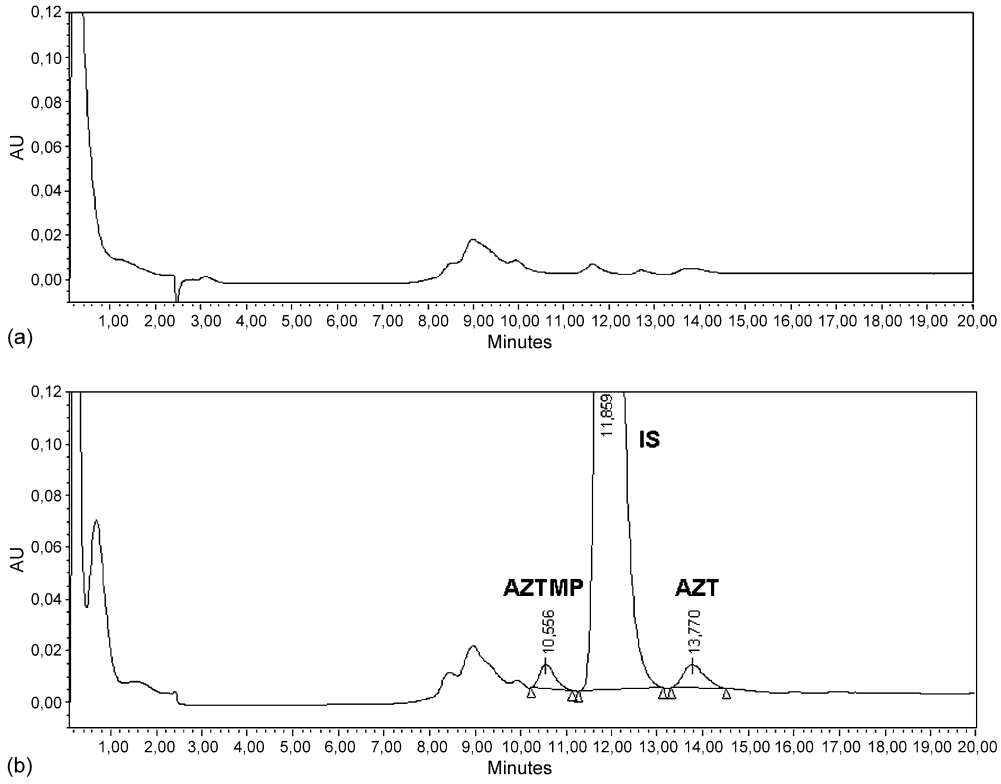

Fig. 4. Typical chromatogram of drug free cells extracts (a) and cell extracts spiked with AZT, AZTMP and IS at a concentrations of 0.5 μg/ml for AZT and AZTMP) and 50 μg/ml for IS (b).

compounds show little or no retention on traditional reversed-phase columns. When water was used as mobile phase, AZTMP eluted from the precolumn near the solvent front.

Thus, a reverse phase HPLC approach with addition of an ion-pairing reagent to the mobile phase was developed to enhance retention of AZTMP on the precolumn and to alter the chromatographic selectivity of AZT, AZTMP and the internal standard on the analytical column. Various positively charged ammonium ions with different alkyl chains (ammonium acetate, triethylammonium acetate, tetrabutylammonium hydrogen sulfate) were investigated to study the influence of the ion-pairing agent hydrophobicity on the retention of AZTMP on the precolumn. When using tetrabutylammonium hydrogen sulfate, AZT was not retained on the precolumn while it was retained when ammonium acetate and triethylammonium acetate were used.

Table 1
Recovery of AZT and AZTMP in cell extracts

Concentration added (μg/ml)	Recovery	R.S.D. (%)
AZT		
0.5	97.2 ± 9.2	±9.4
50	98.2 ± 1.9	±1.9
100	104.7 ± 2.2	±2.1
AZTMP		
0.5	80.6 ± 2.6	±3.2
50	96.6 ± 2.8	±2.9
100	103.6 ± 2.3	±2.2

Triethylammonium acetate salt gave the best results, allowing AZTMP and AZT to be both retained on the precolumn.

Triethylammonium acetate salt concentration was varied in the range 0–50 mM to optimise the separation of AZT, AZTMP and IS on the analytical column. Optimal separation was achieved with 20 mM triethylammonium acetate salt in the mobile phase. Better selectivity was obtained when using acetonitrile as the organic modifier in eluent B compared to methanol.

3.2. Method validation

3.2.1. Stability of stock solutions

Stability of stock solutions was studied over a period of 30 days. Solutions were kept at −20 °C. No degradation of AZT (97.8 ± 5.0% remaining), AZTMP (102.5 ± 10.2% remaining) and IS (101.0 ± 4.2% remaining) was observed over this period.

3.2.2. Stability in cells extract

The stability of AZTMP, AZT and IS in cell extracts and in cell extracts containing phosphatase inhibitor cocktails 1 or 2 was assessed by analyzing samples exposed for different times at 37 °C (Fig. 3).

The decomposition kinetics of AZTMP was first studied in total CEM-SS cell extracts by characterizing an enzymatic dephosphorylation of AZTMP into AZT. The half-life of this compound was 3.7 h.

Then, the decomposition kinetics of AZTMP in cell extracts containing phosphatase inhibitor cocktail 1 or 2 were studied. In cell extracts containing phosphatase inhibitor cocktail 1, the half-life of AZTMP obtained was close to the one observed in total CEM-SS cell extracts. When the phosphatase inhibitor cocktail 2 was added to cell extracts, the half-life of AZTMP was four times longer ($t_{1/2}$ = 14.5 h). The IS and AZT were stable over the studied period in cell extracts with and without phosphatase inhibitors. Consequently, all experiments were performed using cell extracts previously incubated with phosphatase inhibitor cocktail 2.

3.2.3. Specificity

The specificity of the method was assessed by comparing the chromatogram of a standard solution containing AZT (0.5 μg/ml), AZTMP (0.5 μg/ml) and IS (50 μg/ml) with the chromatogram of several sources of blank cell extract (n = 6) (Fig. 4). Blank cell extracts were free of interferences at the retention times corresponding to the compounds of interest.

3.2.4. Recovery

The recovery of AZT and AZTMP in cells extracts was evaluated at three levels of concentration, 0.5, 50 and 100 μg/ml. The mean extraction recoveries were 97.2, 98.2 and 104.7% for AZT and 80.6, 96.6 and 103.6% for AZTMP, respectively (Table 1).

3.2.5. LLOQ and LOD

The lower limit of quantification was found as 0.5 μg/ml for both AZT and AZTMP. At this concentration level, the R.S.D. was less than 12% and the bias was less than 14% for both compounds (Table 2), which fulfills the recommendations for bioanalytical method validation [17].

The LOD (signal/noise = 3) was estimated as 0.5 and 0.1 μg/ml for AZT and AZTMP, respectively.

3.2.6. Linearity

Statistical study of the results (ANOVA) revealed that the calibration curves for AZT and AZTMP were linear over the range 0.5–100 μg/ml. For both calibration curves, the intercept was statistically equal to zero. The equations of the lines curves were the following:

$$Y_{AZT} = 0.0004(\pm 0.0120) + 0.0331(\pm 0.0002)X, \quad r^2 = 0.9994$$

$$Y_{AZTMP} = 0.0061(\pm 0.0059) + 0.0166(\pm 0.0001)X, \quad r^2 = 0.9988$$

3.2.7. Accuracy and precision

The intra- and inter-day accuracy and repeatability of the analysis procedure for cell extract samples were evaluated at 0.5, 50 and 100 μg/ml for AZT and AZTMP. Intra-day results were estimated from the analysis of six independently prepared cell extracts on the same day. Inter-day results were obtained from the analysis of three independently prepared cell extracts during three different days. Inter-day accuracy and precision were calculated using one-way ANOVA analysis. The R.S.D. was less than 12% at the LOQ and less than 3% at other levels for both compounds. The bias was less than 14% (Table 2). These results were satisfying [17] since accuracy and precision were within ±15%.

3.3. Application to the degradation kinetics of an AZT pronucleotide

The procedure was used to study the decomposition pathway of a model pronucleotide (Fig. 1) incubated in CEM-SS cell extracts. 50 μl of a stock bis(*t*BuSATE)AZTMP phosphotriester solution (0.635 mg/ml prepared in DMSO) and 50 μl of internal standard solution (1 mg/ml) were diluted to 1 ml with CEM-SS cell extracts in an ice bath. Sterile procedures were used throughout. The solutions were vortex-mixed then divided among HPLC autosampler conic inserts. The inserts were tightly sealed and incubated at 37 °C for the required time and then frozen. Just before HPLC analysis, each insert was quickly thawed, vortex-mixed and 80 μl of solution were analyzed without further treatment.

The results showed that the phosphotriester gave rise to 3.1, 4.5, 7.8 and 6.7 μg/ml of AZTMP after 6, 8, 23.5 and 32.2 h, respectively of incubation in cell extracts according to the decomposition pathway previously described [6]. A repre-

Table 2
Intra-day accuracy and precision for the determination of AZT and AZTMP in cell extracts

Concentration added (μg/ml)	Intra-day			Inter-day		
	Concentration found (μg/ml)	Bias (%)	R.S.D. (%)	Concentration found (μg/ml)	Bias (%)	R.S.D. (%)
AZT						
0.5	0.48 ± 0.04	−4.0	±8.33	0.43 ± 0.01	−14.0	±8.00
50	54.92 ± 0.21	+9.8	±0.38	52.37 ± 0.38	+4.7	±0.72
100	102.68 ± 2.51	+2.7	±2.44	101.99 ± 1.65	+2.0	±1.61
AZTMP						
0.5	0.44 ± 0.05	+12.0	±11.36	0.45 ± 0.02	−10.0	±6.44
50	55.57 ± 0.31	+11.2	±0.55	56.17 ± 0.34	+12.3	±0,60
100	103.23 ± 2.14	+3.2	±2.08	101.99 ± 3.03	+2.0	±2.97

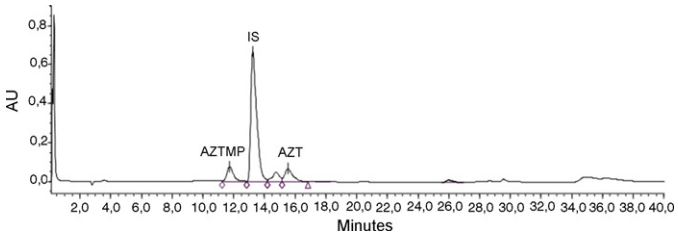

Fig. 5. UV chromatogram obtained after 23.5 h incubation of (*t*BuSATE)AZTMP phosphotriester in cell extracts at 37 °C.

sentative chromatogram obtained after 23.5 h of incubation of the prodrug (initial concentration 31 μg/ml) is given in Fig. 5.

4. Conclusion

An HPLC method using on-line extraction has been developed for the determination of AZT and AZTMP in cell extracts. The method reported is simple, rapid and reproducible. The methodology described was used to measure the concentration of AZTMP and AZT obtained after incubation of a prodrug in cell extracts.

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