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# HPLC-MONITORING OF AZT IN HIV-INFECTED PATIENT'S PLASMA: A CRITICAL STUDY

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*Summary.* – 3'-azido-2', 3'-deoxythymidine (AZT) concentrations in spiked human plasma were determined by means of reversed-phase high performance liquid chromatography (RP-HPLC). Samples were first cleaned-up for analysis using solid-phase extraction (SPE) columns filled with Silipore C18. In the concentration range comprising usual peak plasma concentrations during AZT therapy (0.1–20 μmol/l, i. e. 0.026–5.34 μg/ml) mean efficiency of the extraction procedure reached as high as 75.3 % of original AZT concentrations in standard unextracted aqueous solutions. Replicate analyses in this range gave satisfactory intra-assay precision and reproducibility with coefficient of variation less than 11.3 %. Calibration curves both in water and plasma showed good linearity ($r>0.999$). The detection limit in plasma was 2 μmol/l, i. e. 5.3 ng per a 20 μl of sample injected to the HPLC column. Plasma levels of AZT after a single dose administration, determined by HPLC and RIA showed rather poor correlation ($r=0.8900$). In RIA about 1.7–4.5 times higher concentration values were obtained in a relatively short time, and, consequently, this method may better fulfil the needs of routine drug monitoring.

*Key words: AZT-monitoring; reversed-phase HPLC; HIV*

## *Introduction*

The azido-substituted derivative of thymidine (AZT) is the first and hitherto only licensed drug in treatment of human immunodeficiency virus (HIV) infection (for review see: Stretcher, 1989), the primary cause of acquired immunodeficiency syndrome (AIDS). Its clinical efficacy against HIV-disease (Chaisson *et al.*, 1986; Fischl *et al.*, 1987), as well as its pharmacokinetics, bioavailability, and therapeutic range (i. e. *in vivo* correlation between drug levels, efficacy, and toxicity) in humans have been established (Klecker *et al.*, 1987). However, the ability to absorb and eliminate AZT shows rather high

interpatient variability, especially in those with intestinal disturbances, renal and/or hepatic failure (Blum *et al.*, 1988) and, consequently, to adjust the AZT dosage properly, the drug monitoring is warranted. Currently there are two commonly used methods for measurement of plasma AZT concentration: HPLC (Good *et al.*, 1988; Underberg *et al.*, 1989; Kamali and Rawlins, 1990; Hedaya and Sawchuk, 1990) and radioimmunoassay (RIA) (Quinn *et al.*, 1989; Koppen *et al.*, 1989). More recently a fluorescence polarization immunoassay (FPIA) reported by Stretcher *et al.* (1989), and an enzyme-linked immunosorbent assay (ELISA) and time-resolved fluoroimmunoassay (TR-FIA) (Tadepalli and Quinn, 1990) have been introduced. Although each of the above mentioned approaches have their own benefits as well as disadvantages, experience with HPLC and RIA has more frequently been discussed (Stretcher, 1989; Batra *et al.*, 1989; Tadepalli and Quinn, 1990).

In this communication we report about an RP-HPLC method used for determination of AZT concentration in standard spiked plasma, aqueous solutions, and representative samples of an HIV-infected patient's plasma collected in different time periods following a single dose (200 mg) AZT administration orally. Prior to HPLC analysis, all standards and samples have been extracted with chloroform and subjected to SPE on Silipore C18 columns prepared in our laboratory. The recovery of AZT after SPE as a percent of the corresponding concentrations obtained from the HPLC analysis of unextracted aqueous solutions is given and evaluated with respect to the data obtained by RIA.

## *Materials and Methods*

*Preparation of standards.* Three sets of stock solutions of AZT (98.3 %, w/w) (Lachema, Brno) in water were prepared by sequential dilution in the following concentration ranges: 500-62.5, 100-6.25, and 10-0.625 $\mu$g/ml.

Samples of plasma obtained from healthy volunteers were spiked with AZT and serially diluted with normal plasma in the same concentration ranges. About 30 ml of each standard were prepared, divided into 1.1-ml aliquots and stored at -20 °C. Plasma samples from an asymptomatic HIV-1 seropositive patient receiving orally a single dose of 200 mg of AZT (in tablets from Lachema, Brno) were collected at 1, 10, 20, 30, 45, 60, 90, 120, and 180 minutes after the drug administration. 1.1 ml replicates of each sample were stored at -70 °C until analysed.

*Sample clean-up and SPE.* Prior to SPE 5 replicates of each standard and 2 replicates of samples have been extracted for 10 min with chloroform in a ratio of 5:1 (v/v) and centrifuged at 3500 rpm in a T62 centrifuge (VEB MLW Zentrifugenbau, Engelsdorf, Germany) for 10 min. This essential step removes interfering lipophilic substances deteriorating the performance of HPLC column. 0.5 ml of clear upper phase was diluted with an equal volume of 0.05 mol/l $KH_2PO_4$, pH 8.5 and loaded onto the preconditioned SPE column. The columns were home-made from 1000 $\mu$l micropipette tips and filled with 100±10 mg of Silipore C18 (d(p): 125–160 $\mu$m, S: 245 m$^2$/g, C: 13.3 % Lachema, Brno). Before loading the samples each column had been washed very slowly by 2 ml of degassed isopropyl alcohol followed by 2 ml of degassed 0.05 mol/l $KH_2PO_4$, pH 8.5. The samples were then allowed to equilibrate and suck in for at least 5 min and the columns were washed by 1 ml of degassed 0.05 mol/l $KH_2PO_4$, pH 8.5. Up to this point the SPE columns were not allowed to become dry. In the next step reduced pressure was applied to the columns for about 15–20 min, until they became air-dried. Schleicher and Schuell Filtration Manifold (Dassel, Germany) connected with water jet pump had

been arranged for this purpose. Elution of AZT was effected by 0.5 ml of 80 % (v/v) methanol in water and the eluate was evaporated in vacuum drying oven at 50 °C to dryness. The dry residue was redissolved in 250 μl of 5 % (v/v) methanol in water (this means a two-fold preconcentration), stirred for 5 min and centrifuged (3500 rpm/10 min). 20 μl aliquots of the clear supernatant were injected for RP-HPLC analysis.

*RP-HPLC.* The used computer (PU 3203)-aided Philips (Pye Unicam, Cambridge, England) chromatographic system consisted of a double piston pump with overlapped delivery strokes, PU 4100 liquid chromatograph, PU 4021 multichannel (diode array) UV detector, a Rheodyne (California, U. S. A.) injection valve (model No. 7125), and a reversed-phase C18 column (Separon SGX C18, 7 μm, 15 cm × 3 mm, TESSEK, Prague, Czechoslovakia). Filtration of solvents was carried out using 0.4 μm Synpor filters (VCHZ Synthesia, Pardubice-Semtin, Czechoslovakia) and the mobile phase was degassed by helium (purity 99.9999 %, Messer Griesheim, Gumpoldskirchen, Austria). Prior to analysis the HPLC column had been equilibrated for at least 30 min. Sample elution was isocratic using a mobile phase of 20:80 (v/v) mixture of methanol - $KH_2PO_4$ (50 mmol/l, pH 8.5) at a constant flow rate of 1 ml/min at ambient temperature. The absorbance of the effluent was monitored at 267 nm.

## *Results and Discussion*

### *Recoveries from the extraction columns*

In order to determine the efficiencies of SPE columns used, aqueous and normal plasma solutions of AZT were identically prepared at 4–5 different concentrations in three concentration ranges (Table 1). Five replicates of the plasma solutions were extracted and analyzed. The mean recovery was calcula-

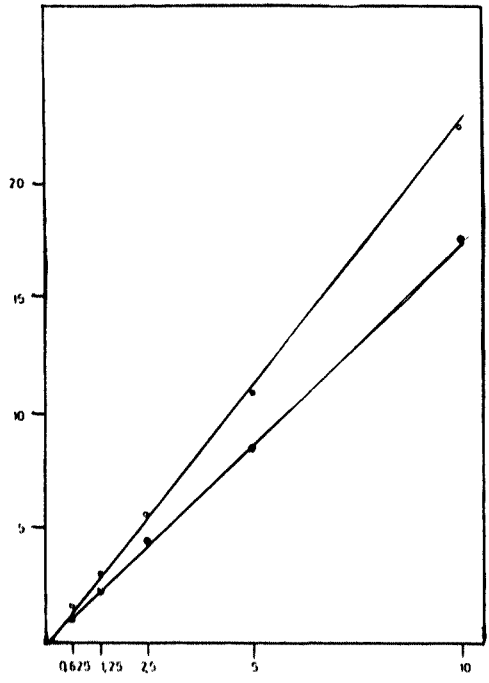


**Fig. 1**
Standard curves for the HPLC analysis of AZT in water and human plasma
Standard aqueous solution and spiked plasma solution of AZT were serially diluted with water and normal plasma, respectively, and five replicates of each concentration were analysed by HPLC. Prior to analysis, SPE of individual spiked plasma samples was carried out. Each point in the standard curves represents mean of five estimations.
Abscissa: concentration of AZT in water (○) and plasma (●) (μg/ml). Ordinate: $A_{267} \times 10^3$.

**Table 1. Intra-assay precision and reproducibility of AZT determination by RP-HPLC**

| Concentration of added AZT (μg/ml) | Injected amount of AZT in a 20 μl volume (ng) | $A_{266} \times 10^3$ | |
|---|---|---|---|
| | | Aqueous solution of AZT without SPE | Plasma solution of AZT after SPE |
| 500.000 | 10 000.0 | 1 237.0 ± 15.7 (1.7) | 843.1 ± 206.5 (24.5) |
| 250.000 | 5 000.0 | 678.0 ± 15.8 (2.3) | 334.2 ± 17.9 (5.4) |
| 125.000 | 2 500.0 | 357.0 ± 2.1 (0.6) | 174.0 ± 6.7 (3.8) |
| 62.500 | 1 250.0 | 172.0 ± 3.7 (2.1) | 80.1 ± 2.4 (3.0) |
| 100.000 | 2 000.0 | 269.0 ± 4.3 (1.6) | 135.9 ± 7.6 (5.6) |
| 50.000 | 1 000.0 | 130.0 ± 2.5 (1.9) | N. D. |
| 25.000 | 500.0 | 63.0 ± 0.5 (0.7) | 31.7 ± 3.4 (10.6) |
| 12.500 | 250.0 | 31.0 ± 0.5 (1.5) | 20.0 ± 0.9 (4.6) |
| 6.250 | 125.0 | 12.0 ± 0.0 (0.0) | 8.8 ± 1.6 (18.4) |
| 10.000 | 200.0 | 22.6 ± 0.8 (3.9) | 17.3 ± 0.4 (2.3) |
| 5.000 | 100.0 | 11.0 ± 0.1 (1.1) | 8.6 ± 0.5 (5.5) |
| 2.500 | 50.0 | 5.9 ± 0.1 (1.4) | 4.4 ± 0.3 (7.5) |
| 1.250 | 25.0 | 2.9 ± 0.05(1.6) | 2.1 ± 0.05 (2.2) |
| 0.625 | 12.5 | 1.6 ± 0.0 (0.0) | 1.1 ± 0.1 (11.3) |

Values given are mean (n=5) ± S. D. In parentheses percent coefficients of variation are given. N. D.: not determined

ted by comparing observed AZT concentrations with those obtained from direct injection of standard unextracted aqueous solutions (Table 2). Mean recoveries of AZT in all three concentration ranges were higher than 53 %, reaching in the lowest concentration range as much as 75.3 %. The reproducibility of the recoveries was the best in the lowest concentration range as well, with all

**Table 2. Mean recoveries of AZT after SPE in three different concentration ranges**

| Concentration range (μg/ml) | $A_{266} \times 10^3$ | | Efficiency of SPE (%) (range) |
|---|---|---|---|
| | Aqueous solution of AZT without SPE | Plasma solution of AZT after SPE | |
| 500 - 62.500 | 1 237.0 ± 172.0 | 843.1 ± 80.1 | 53.2 (46.6-68.2) |
| 100 - 6.250 | 269.0 ± 12.0 | 135.9 ± 8.8 | 59.7 (50.3-73.3) |
| 10 - 0.625 | 22.6 ± 1.6 | 17.3 ± 1.1 | 75.3 (68.8-82.4) |

Values given are mean ± S. D.

coefficients of variation less than 11.3 % (Table 1). This observation is seemingly controversal to that of Good *et al.* (1988) who found both, higher mean recoveries and better reproducibility at higher plasma concentrations of AZT. However, the lowest concentration range in our analysis (0.625–10 μg/ml) does correspond to the upper half of concentration values (2–20 μmol/l) tested by these authors.

*Standard curves*

At the beginning of our assay a five-point standard curve for each concentration range of AZT both, in unextracted aqueous solution and solid-phase extracted normal plasma was generated. Fig. 1 demonstrates the standard curves for concentrations 0.625–10 μg/ml, most often observed in peak plasma levels during pharmacokinetic studies, plotted as $A_{267}$ versus concentration. Although the calibration curves for both, aqueous solution and plasma gave good linearity ($r>0.999$), the limit for the analyte detection in solid-phase extracted plasma was about 2 μmol/l. This sensitivity is by about one order of magnitude lower than it has been reported by others (Blum *et al.*, 1988; Good *et al.*, 1988; Underberg *et al.*, 1989). According to our experience, at this concentration baselines were still flat and peaks sharp enough to identify AZT but accurate quantification could not be achieved.

*RIA versus SPE/RP-HPLC: comparison of results obtained from a representative*

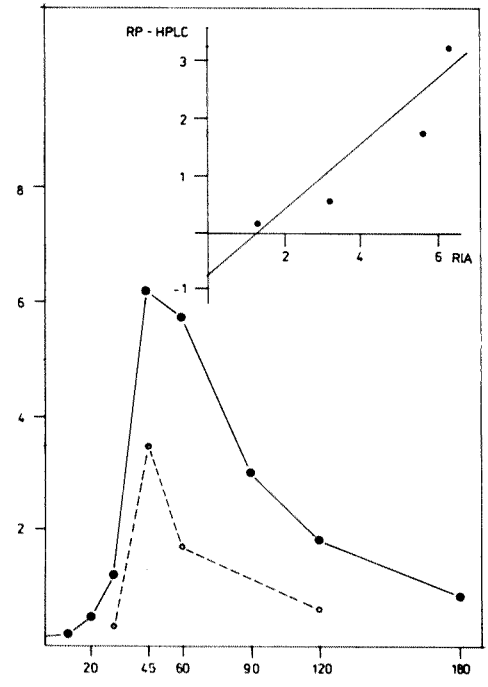


**Fig. 2**
Plasma concentration - time profile of AZT following oral administration of 200 mg of drug
Abscissa: time of sample collection (min). Ordinate: plasma concentration of AZT determined by RIA (●) and HPLC (○) (μg/ml). Each point represents mean of two estimations. Insert: correlation between RIA and HPLC in quantitating AZT concentration in plasma samples. Slope = 0.5618, y intercept = -0.7147, r = 0.8900, n = 4.

*case of drug monitoring*

Concentration of AZT in eight plasma samples from an HIV-infected patient were obtained by both, RIA (Földeš O., personal communication) and the described SPE/RP-HPLC method, in indicated time periods after a single dose (200 mg) administered orally. Fig. 2 shows the comparison of "plasma concentration-time" profiles. The insertion demonstrates linear regression plotting of RP-HPLC values versus those obtained by RIA. It is obvious that in RIA about 1.7–4.5 times higher concentration values were repeatedly obtained, and that there is a rather poor correlation between these two methods ($r=0.8900$). This observation is in good accordance with other reports (Batra *et al.*, 1989; Földeš O., personal communication) and underlines the priority of RIA for routine drug monitoring, especially when large number of samples should be analyzed in a relatively short time. New methods reported recently by Tadepalli and Quinn (1990) mean further straightforward step in this direction. On the other hand, this time only HPLC allows direct concurrent determination of AZT and its major hepatic conversion product, the 5'-O-glucuronide (3'-azido-3'-deoxy-5'-$\beta$-D-glucopyranuronosylthymidine, GAZT).

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