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# PLASMA LEVELS OF THE ANTI-HIV DRUG 3'-AZIDO-2', 3'-DIDEOXYTHYMIDINE (AZT): DETERMINATION BY RIA AND HPLC

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*Summary.* – The radioimmunoassay (RIA) with two commercial kits and the high pressure liquid chromatography (HPLC) method were employed to test human and dog blood plasma levels of AZT following oral and intravenous administration of AZT preparations Retrovir[R] and Azitidin. A comparison of results obtained by the two RIA kits showed a good correlation. A weaker correlation was established in comparing the results obtained by RIA with those obtained by HPLC. Because of its reliability, rapid availability of results and the price, RIA is more advantageous than HPLC for routine use in monitoring the therapy as well as in determining pharmacokinetic parameters of AZT.

*Key words: azidodideoxythymidine; plasma level; RIA; HPLC; HIV*

## *Introduction*

AZT (Azitidin, Retrovir[R], Zidovudine, ZDV) is a synthetic analogue of thymidine which effectively inhibits the replication of human immunodeficiency virus (HIV). AZT is phosphorylated by thymidine kinase and other intracellular enzymes into AZT-triphosphate which interact in infected cells preferentially with HIV reverse transcriptase, and this results in premature termination of proviral DNA synthesis (Furman *et al.*, 1986).

So far, the first compound from the group of dideoxynucleoside analogues inhibiting HIV replication by a similar mechanism and employed clinically has been AZT (Mitsuya *et al.*, 1986). A significant decrease of opportune infections and death rates (as compared to a group of patients given placebo) were demonstrated in extensive clinical studies in patients with AIDS given AZT (Yarchoan *et al.*, 1986; Fischl *et al.*, 1987). A decrease of HIV antigen p24 levels in serum and cerebrospinal fluid following AZT administration suggests some association between AZT administration, inhibition of HIV replication and improvement of clinical condition of AIDS patients (De Gans *et al.*, 1988).

AZT concentrations which effectively inhibited HIV-1 replication were studied predominantly in *in vitro* experiments. However, the results published so far cannot always be compared with each other due to differences in experimental models used by the investigators. The report most frequently referred to, has been that by Mitsuya *et al.* (1985) who demonstrated inhibition of expression of viral antigens in HIV-1 infected lymphoblastoid cell cultures at 1 μmol/l AZT. Other investigators reported lower inhibitory concentrations obtained from *in vitro* experiments (Pauwels *et al.*, 1987). It has been generally accepted that the administration of the drug for therapeutical purposes requires plasma concentrations not below 1 μmol/l (Yarchoan *et al.*, 1989). Although a therapeutical "window" for AZT has yet to be described, it is already known that plasma concentrations of AZT achieved using the dosage schedule advised by the drug manufacturer vary individually. Different pharmacokinetic parameters for AZT were reported in some risk groups (Morse *et al.*, 1989). The therapeutic usefulness of AZT is limited mainly by the toxicity of the drug. Particularly neutropenia resulting from bone marrow depression and overall haematological toxicity, as well as the need of maintaining a minimal plasma concentration of AZT over prolonged intervals support the requirement of regular check-ups of AZT plasma concentrations both at the start of and during the treatment with AZT.

HPLC (Good *et al.*, 1988; Unadkat *et al.*, 1988) and a group of immunochemical methods (RIA, EIA etc., Quinn *et al.*, 1989; Tadepalli *et al.*, 1990*a*) are presently being employed for the determination of plasma AZT concentrations. The present study was conduced to compare AZT levels measured in dog and human plasma using commercial RIA kits with those measured by HPLC. The RIA was shown to provide sufficiently sensitive, specific and reproducible results over the entire range of levels tested; this technique may therefore be recommended for the determination of pharmacokinetic parameters of AZT as well as for the monitoring of the drug concentrations during the treatment. Plasma analyses with the HPLC yield less satisfactory results.

## *Materials and Methods*

*AZT preparations.* 1. Retrovir$^R$ (Burroughs Wellcome Co., Research Triangle Park, U. S. A), 100 mg per capsule for peroral application. 2. Azitidin (Lachema Brno, Czechoslovakia), 100 mg per tablet. For intravenous administration, 1 % solution of Azitidin in sterile saline was prepared from recrystallized preparation.

*Patients.* Twelve patients (only men) were enrolled to the study after they gave informed consent. All of them were HIV seropositive as confirmed by positive Western blot tests. They were asymptomatic from their HIV infection except two with ARC (AIDS - related complex). Their average age was 35.5 years (range 23 to 51). The patients were not receiving any chronic medication which could interfere with metabolism and/or concentration of AZT. No alterations in their gastrointestinal and/or renal function were observed prior to drug administration. Patients received first single dose of 200 mg of Azitidin perorally and 14 days later a similar dose of Retrovir$^R$. Blood was sampled 0, 10, 20, 30, 45, 60, 90, 120, 180, 300 and 480 min after administration of each drug into heparinized tubes. Plasma was obtained by centrifugation and stored at -70 °C until assayed.

*Animals.* Male beagle dogs were used (b.w. approx. 20 kg). They received intravenous Azitidin, 48.5 mg per animal. Azitidin and Retrovir$^R$ were administered over 14 days in oral doses of 100 mg per animal. Blood samples were taken from the antebrachial cephalic vein at the same intervals as above, and processed in an identical way.

*RIA.* Kits for AZT: 1. $^{125}$I-AZT RIA (PEG) kit (Institute of Isotopes, Hungarian Academy of Sciences, Budapest, Hungary); 2. ZDV-Trac $^{125}$I RIA kit (Incstar Co., Stillwarer, U. S. A.). Both kits were treated as recommended by the respective manufacturer, and all plasma samples were processed in parallel. Human plasma controls containing AZT were prepared by adding small aliquots of Azitidin stock solution to pooled pre-dose samples to give final concentrations 1, 5 and 10 $\mu$g/ml. These samples were analyzed by both RIA kits and results were used for recovery calculations.

*HPLC.* AZT plasma concentrations were determined as described by Good *et al.* (1988) with a slight modification. A HP 1090 Hewlet Packard apparatus was used, equipped with a 4 ×250 mm stainless steel column packed with Silasorb SPH $C_{18}$ (particle diameter 7.5 $\mu$m). The mobile phase (50 mmol/l $KH_2PO_4$: methanol = 80:20 v:v) was run at 1.5 ml/min and AZT was determined at 266 nm.

## *Results*

Table 1 shows the basic parameters of both AZT RIA kits. Obviously, the calibration curves of both kits (in terms of pg/tube) differ, with the $^{125}$I-AZT RIA (PEG) kit having a higher sensitivity; on the other hand, this requires higher dilutions of samples to be analyzed. The instructions to the Hungarian product lack data concerning cross-reactivity with the glucuronide of AZT (3'-azido-3'-deoxy-5'-0-$\beta$-D-glucopyranosylthymidine, ZDVG, GAZT), which is reported to be very low (0.01 %) with the ZDV-Trac $^{125}$I RIA kit. The composition of both kits as well as the procedures are almost identical, but a considerable difference

**Table 1. Parameters of AZT RIA kits**

| Parameter | $^{125}$I-AZT RIA (PEG) kit | ZDV-Trac $^{125}$I RIA kit |
|---|---|---|
| Anti-AZT antibody | rabbit | rabbit |
| Range of calibration curve | 1.23–500 pg/tube (12.3–5000 ng/ml) | 40–52 600 pg/tube (4.2–5523 ng/ml) |
| Sensitivity | 1.5 pg/tube | 53.4 pg/tube |
| Crossreactivity thymidine | < 0.01 | < 0.0005 |
| 2-deoxyuridine | < 0.01 | n.i. |
| GAZT | n.i. | 0.01 |
| Recovery | 93–107 % | 91–102 % |
| Total activity | 20 000 cpm/tube | 38 000 cpm/tube |
| Dilution of samples | 1:1000 | 1:21 |
| Separation system | polyethyleneglycol | goat anti-rabbit antibody + polyethyleneglycol |
| Nonspecific binding | 8 % | 1.2 % |

n.i. = no information

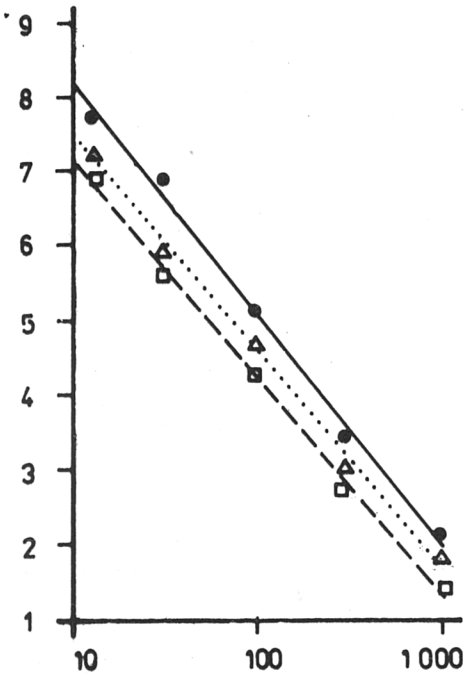


**Fig. 1**
The effect of human plasma on the calibration curve of $^{125}$I-AZT RIA (PEG) kit
10 $\mu$l of plasma (final dilution – 1:10 000) or standard buffer were added to the tubes for calibration curve. Abscissa: AZT (ng/ml); ordinate: cpm × 1000.
△ regression curve with human plasma,
□ regression curve with dog plasma,
● regression curve with buffer.

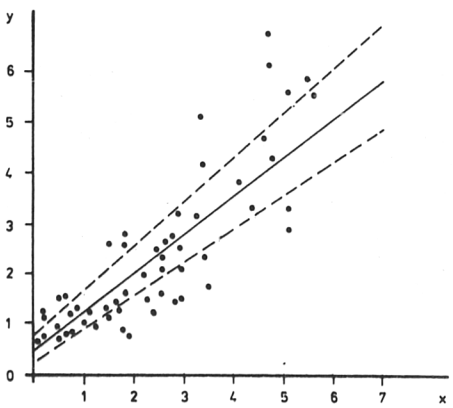


**Fig. 2**
Correlation between AZT concentrations in human plasma samples determined by two RIA kits
x – plasma levels of AZT ($\mu$g/ml) by $^{125}$I-AZT RIA (PEG) kit; y – plasma levels of AZT ($\mu$g/ml) by ZDV-Trac $^{125}$I RIA kit; $y = 0.765x + 0.523$ (continuous line); $y \pm 2SD = 0.765 (x \pm 2SD) + 0.523$ (broken line); correlation $r = 0.899$.

is in the length of incubation (2 hr with the ZDV-Trac $^{125}$I RIA kit, and 16–20 hr with the $^{125}$I-AZT RIA (PEG) kit). In spite of the high dilution of samples to be analyzed, the Hungarian manufacturer points to possible "protein effect". Indeed, if dog plasma diluted 1:10 000 was added into the tubes for calibration curve, a shift toward lower values was obtained. A similar, though less pronounced effect was observed with human plasma (Fig. 1). The manufacturer of the ZDV-Trac $^{125}$I RIA kit does not recommend plasma addditions into the tubes for calibration curve; moreover, as it is not clear from the instructions whether or not the standards contain plasma, we did not check this effect with this kit.

Both kits were used to determine AZT concentrations in human plasma samples in patients following oral administration of Retrovir$^{R}$ and Azitidin. Fig. 2 shows a comparison of AZT concentrations in human plasma as measured by the two RIA kits. The values obtained show a relatively good correlation. Greater differences occur within the low AZT concentrations range (less than 1 $\mu$g/ml). Using the Hungarian kit, AZT plasma levels at the sensitivity level could be measured in both the dog and human plasma samples taken before the drug administration; whereas in the same samples the ZDV-Trac $^{125}$I RIA kit gave values equal to or exceeding 0.3 $\mu$g/ml.

The dog plasma samples used to determine AZT levels by RIA ($^{125}$I RIA(PEG) kit) were also tested by HPLC. A comparison of the results obtained using Hungarian RIA kit with those obtained by the HPLC is shown in Fig. 3. The correlation is not as perfect as that between the two RIA kits. The phenomenon similar to that illustrated in Fig. 2 could be observed in the low concentration range.

Plasma AZT levels in one of the patients following oral AZT administration

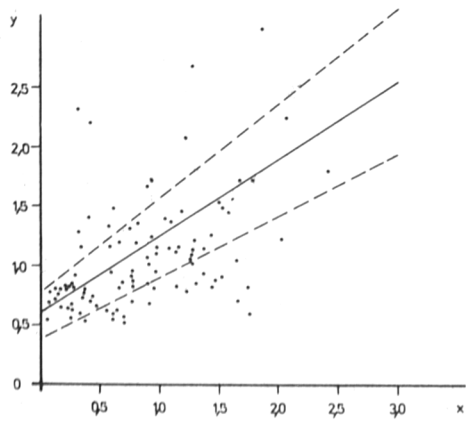


**Fig. 3**
Correlation between AZT concentrations in dog plasma samples determined by HPLC and RIA
x – plasma levels of AZT ($\mu$g/ml) by $^{125}$I-AZT RIA (PEG) kit; y – plasma levels of AZT ($\mu$g/ml) by HPLC; $y = 0.68x + 0.54$ (continuous line); $y \pm 2SD = 0.68 (x \pm 2SD) + 0.54$ (broken line); correlation $r = 0.817$.

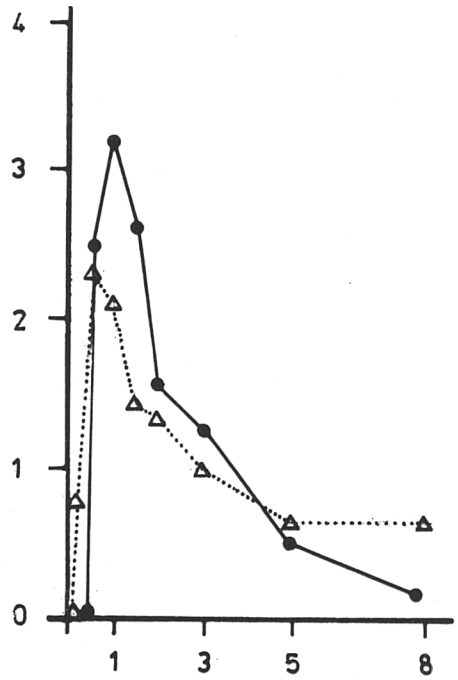


**Fig. 4**
AZT plasma concentration determined by two RIA kits: time profile in patient No. 2 Abscissa: time (hr); ordinate: AZT (μg/ml). ●$^{125}$I-AZT RIA (PEG) kit, ▽ ZDV-Trac $^{125}$I RIA kit.

(200 mg of Retrovir) determined by $^{125}$I-AZT RIA (PEG) kit, as well as by ZDV-Trac $^{125}$I RIA kit, is shown in Fig. 4.

## *Discussion*

AZT was the first drug which passed with effort clinical trials and received FDA approval for use in patients with AIDS in U. S. A. However, several questions remain unresolved. Initially, the starting dose 250 mg orally every 4 hr was recommended because of the very short half-life (approx. 1 hr) (Yarchoan *et al.*, 1986), but it caused adverse reactions associated with AZT therapy observed in some patients receiving 1500 mg total daily dose (Richman *et al.*, 1987). Lower doses are in general better tolerated, and it seems that the clinical effect is satisfactory also under similar conditions (Fischl, 1988; Volberding *et al.*, 1990). Another way of AZT dosage reduction by means of regular therapy breaks (intermittent therapy) was introduced by Staszewski *et al.* (1991). On the other hand, pharmacokinetic studies have shown wide range of peak concentrations obtained following the oral administration of a standard dose of AZT (Morse *et al.*, 1989; Blum *et al.*, 1988; Balis *et al.*, 1989). This may result from interindivi-

dual differences in absorption or metabolism of the drug (Taburet *et al.*, 1990). Because of impaired AZT absorption, careful evaluation of low AZT regimens previously considered efficacious is required e.g. in patients with AIDS-related small intestinal disease (Kapembwa *et al.*, 1991). So, reduced single doses or changed dosing interval may, in some subjects, decrease the levels of AZT below the concentration which *in vitro* inhibits HIV replications, whereas in other subjects it need not. When changing AZT regimens it seems therefore necessary to check AZT levels inddividually to find the optimal therapeutic ratio.

Two basic methodological approaches to AZT determination in biological material are available: the group of immunoassays, and HPLC. A number of reports have been published concerning both techniques. The present study was undertaken to compare the results obtained using two commercial kits with those obtained by HPLC determination. Two preparations containing AZT were tested in dogs and humans: Retrovir$^{R}$ and Azitidin. The RIA kits employed were processed exactly as recommended by the respective manufacturer, the HPLC technique was modified according to the capacity of the laboratory. Basic pharmacokinetic parameters of both preparations tested were computed from the AZT concentrations measured.

As far as the same sample was analyzed using both kits, the results showed less satisfactory coincidence. Results obtained using both RIA kits agree only partially: both kits in parallel measured high, medium or low concentrations. Less satisfactory was mutual correlation of the actual values. Some difficulties occurred with the determination of AZT concentrations in samples taken before the drug administration or in those containing low AZT levels. For these samples the Hungarian kit usually gave zero values or values close to the lowest detectable concentration. The presence of diluted plasma seems to interfere with the RIA though both kits require diluted plasma samples. The instructions to the Hungarian kit account for this fact, and we could confirm this experimentally.

Within the range of medium and high concentrations, the Hungarian kit gave somewhat higher AZT values than did the Incstar kit. We can speculate that the higher values obtained might have been due to cross-reactivity of the antibody contained in the kit with some of the AZT metabolites (most probably GAZT); unfortunately, there are no hints as to this possibility in the instructions.

A clear advantage of the ZDV-Trac $^{125}$I RIA kit is that it allows AZT and GAZT determinations in serum and urine samples (Tadepalli *et al.*, 1990*b*). As far as the complexity is concerned, both kits are equally good. However, the ZDV-Trac $^{125}$I RIA kit is more advantageous for routinè use from the viewpoint of easy and rapid obtaining of results. Considering all advantages and disadvantages of both RIA kits tested, it may be stated that both kits are suitable for studying AZT pharmacokinetics and/or therapy monitoring.

The comparison of results obtained using RIA with those obtained by HPLC is less satisfactory. In addition, AZT plasma concentration in an HIV seropositive patient were analyzed by both RIA kits and compared to the results obtained

by HPLC (Borvák *et al.*, 1992). Better correlation was obtained for $^{125}$I RIA(PEG) kit vs. HPLC comparison (correlation coefficient r = 0.865 compared to r = 0.807 for ZDV-Trac $^{125}$I RIA kit vs. HPLC). AZT levels measured in the same samples using the two techniques showed worse correlation than those obtained by the two RIA kits: moreover, even extreme differences occured. The present results show that it is rather difficult to determine "zero" AZT plasma level. Similar problems were reported also by other investigators. Thus, Batra *et al.* (1989), reported a RIA vs. HPLC correlation coefficient for "zero" AZT samples of only 0.2, and also the manufacturer or the ZDV-Trac $^{125}$I RIA kit points in the instructions to similar differences within this concentration range. All AZT concentrations measured by RIA seem to be by approx. 1/3 higher than those obtained by HPLC. We have no explanation for this observation. In addition, HPLC determinations are markedly time and cost demanding. To our experience, both tested RIA kits for the determination of serum AZT concentration have greater sensitivity than HPLC, are easier to run and are more time and cost effective. Of the two RIA kits, the Hungarian one provides more adequate results, and has a greater sensitivity. On the other hand, it requires longer incubation time, and lacks information concerning cross-reactivity with AZT metabolites. It lacks the comfort of the Incstar kit, and the high dilutions required make it less practical.

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