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Evaluation of DNA adduction of AZT in peripheral blood leukocytes of HIV-infected individuals by ^{32}P-post-labeling thin-layer chromatography: a feasibility study

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Abstract

3′-Azido-3′-deoxythymidine (AZT, Zidovudine) has been effectively used for HIV infection treatment. It inhibits virus reproduction through viral reverse transcriptase inhibition. However, the side effects of this anti-retroviral drug might be cumulative, particularly in its effects on the patients' DNA. As a nucleoside analogue, AZT might incorporate into hosts' DNA, and then form DNA adducts. This may result in potential long-term risks of mutagenesis in AIDS patients who received therapy. In this feasibility study, a ^{32}P-post-labeling thin-layer chromatography (TLC) assay is successfully used to measure AZT–DNA analogue and adducts formed in peripheral blood leukocytes of AZT treated patients. There are DNA analogue/adducts measured in all four AZT treated patients' DNA specimens. This assay is reliable with the significant coefficient of correlation in both intra-assay (r = 0.8761, P = 0.0001) and inter-assay (r = 0.8761, P = 0.0001).

Keywords: DNA; 3′-Azido-3′-deoxythymidine

1. Introduction

3′-Azido-3′-deoxythymidine (AZT, Zidovudine), as a nucleoside analogue, has been widely used as treatment for HIV infection ever since its approval by the FDA in 1987. It was used first as monotherapy, and more recently in combination with other nucleoside analogues and protease inhibitors, in combinations commonly referred to as HAART (highly active antiretroviral therapy). AZT has been proven effective in the inhibition of retroviral reverse transcription of HIV-1 virus by its preferential interaction with viral reverse transcriptase. AZT is an analogue of thymidine in which an azido group replaces the 3′-hydroxyl group. It is metabolized intra-cellularly through a series of phosphorylation steps to its active form 5′-triphosphate AZT (AZT-TP). AZT-TP competes with dTTP (thymidylate) for retroviral reverse transcriptase enzyme, reducing transcription of HIV RNA to double-stranded DNA and, thereby, reducing viral replication. It also incorporates into the growing viral DNA chain and leads to the termination of DNA elongation for the lack of the 3′-hydroxyl group that is essential in the formation of 5′–3′ phosphodiester linkage. A second metabolite of AZT, 5′-monophosphate AZT (AZT-MP) that accounts for

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approximately 95% of the total AZT phosphate derivatives inside the cell, is known to have cytotoxic and mutagenic effects [1]. It has been shown that AZT-MP suppresses the 3′–5′ exonuclease activity, leading to the impairment of cellular DNA repair [2]. Furthermore, accumulation of high level of AZT-MP in tissues has been suggested to result in decreased formation of the dTTP needed for cellular DNA synthesis [3]. All these raise concerns regarding the potential mutagenicity of AZT in HIV/AIDS patients who are chronically taking the drug.

Nucleoside analogues, such as AZT, might be incorporated into hosts' DNA and form analogues, cause adducts, diminish repair, and perturb purine/pyrimidine metabolism.

Studies in mice have found that concentrations of AZT metabolites vary between tissues, most likely because of differences in rates of phosphorylation of AZT [4,5]. Pharmaceutical sponsored studies showed that intra-vaginal administration of AZT in a lifetime resulted in a high incidence of vaginal squamous cell carcinoma [6]. An additional study similarly in mice and monkeys found that with increased uptake of AZT, there might be increased tumor risk in the lung, liver and other female reproductive organs [7]. In Olivero's [8] study, 86% of 28 AZT-exposed HIV-infected individuals tested positive for AZT–DNA adducts in an AZT radioimmunoassay (RIA).

DNA adducts and analogues are formed by chemical reactions between genomic DNA and exogenous molecules, such as cisplatin [9] and insertion of exogenous molecules, e.g., bromouracil [10]. They are commonly used as biomarkers of risk in people because of their associations with genomic instability and association with development of tumors in animal models. Levels of DNA adducts vary depending on the extent and frequency of the exposure, the rate of xenobiotic metabolism and the rate of DNA adduct repair. In these studies, we successfully demonstrate the assay feasibility to examine the perturbation of AZT in DNAs of HIV-infected individuals by the ^{32}P-post-labeling and two-dimensional thin-layer chromatography (2-D TLC).

2. Experimental

2.1. Nucleic acids

Calf thymus DNA (Type I; highly polymerized), 2′-deoxy-5′-nucleotides (dAMP, dCMP, dGMP, dTMP—deoxynucleotide monophosphates; dATP, dCTP, dGTP, dTTP—deoxynucleotide triphosphates), as cold phosphate controls, were purchased from Sigma Chemicals, Inc. (St. Louis, MO, USA). Alpha-^{32}P-radiolabeled (3000 uCi/mmol; 8–16 uCi per "nick") dATP, dCTP, dGTP, and dTTP were purchased from Amersham, Inc., or New England Nuclear (Dupont, Inc.; Cambridge, MA, USA). Transfer ribonucleic acid (tRNA) from Type XXI/*Escherichia coli*, strain W, was purchased from Sigma.

2.2. Enzymes

Micrococcal nuclease (EC 3.1.31.1; activity: 100–200 micromolar units/mg of protein), and spleen phosphodiesterase II (EC 3.1.16.1; activity: 13.5 units/mg of protein) were purchased from Sigma. DNase I, and *E. coli* DNA polymerase I were purchased from Boehringer–Mannheim, Inc. (Indianapolis, IN, USA).

2.3. Preparation of autoradiograms

DNA was extracted from anonymous blood samples (+anonymous control) within 48 h after blood samples were drawn. A buffy-coat fraction from whole blood was prepared by centrifuging whole blood at 3300 × *g* for 10 min, which would increase total DNA yields by 5–10 times. QIAamp DNA mini kit from QIAGEN was used for the DNA extraction. Concentration of DNA was adjusted to 0.66 μg/μl with phosphate buffered saline (PBS). Preparation of autoradiograms: 2 μl of DNA and 2 μl of ^{32}P-labeled dATP, dCTP, dGTP, and dTTP were incubated with enzyme mixture of DNA-polymerase I and DNase I from nick-translation kit (Boehringer–Mannheim) at 15 °C for 35 min. ^{32}P were then incorporated into DNA constituent mononucleotides by 5′-phosphorylation via [^{32}P]-*alpha*-dNTP (deoxynucleotide triphosphates) nick-translation. Three cold ethanol washings meticulously removed unincorporated counts, and the remaining pellet was resuspended with Tris–EDTA (TE) buffer at pH 8.0. Subsequently, enzymatic digestion was performed with 40 μl of spleen phosphodiesterase II (activity, 0.3375 units/μl) (Sigma–Aldrich) and 10 μl of micrococcal nuclease (activity, 0.2–0.4 units/μl) (Sigma–Aldrich) in 40 μl of 20 mM sodium succinate and 8 mM calcium chloride (pH 6.0, 37 °C) for 16 h. After precipitation with 500 μl of acetone and collection of the resulting supernatant, a speed vacuum was used until evaporation to dryness was achieved. Pellets were resuspended in 5 μl of dH_2O. Next, a scintillation count was carried out by adding 1 μl of sample in 1 ml of scintillation fluid to determine the radioactivity of ^{32}P-labeled nucleotides. Amount of samples (up to 4 μl) to be spotted on TLC plate was standardized based on radioactivity of the sample. The monophosphate separation was easily accomplished via two-dimensional (2-D) polyester polyethyleneimine (PEI) cellulose TLC, using one solvent in each dimension. For the first dimension, solvent was prepared in 1.0 M acetic acid, pH adjusted to 3.5 with sodium hydroxide. Second dimension consisted of 5.6 M of diammonium sulfate and 0.035 M of ammonium hydrogen sulfate and 0.12 M of disodium EDTA, pH adjusted to 3.5 with sodium hydroxide. The TLC plates were placed in the first-dimension for 8 h and air-dried and then placed in the second-dimension solvent for 16 h and again air-dried. After chromatography, the plates were loaded into cassettes with Kodak XAR-5 film. The cassette were placed in a −84 °C freezer for 20 h to 10 days depending on the adjustment for under- or overexposure the film to elucidate smaller adducts or to demonstrate

sharper adduct resolution. The film was developed and autoradiograms were obtained.

For the quality control, each DNA specimens are tested twice in same day for the intra-assay comparison. All specimens also were tested in different days for the inter-assay comparison.

2.4. Beckmann laser densitometry computer-assisted analysis of the TLC XAR-5 autoradiograms

Laser densitometer is the product of Beckmann Instruments (New York, NY, USA). This has been as sensitive as scintillation counting and is helpful in determining analogue to nucleotide ratios, retention factor, quantification of proximate adducts to known dNMPs, area of spot exposure, and spot density (on gray scale), which reflects quantity of each labeled phosphate. The ability to graphically superimpose each film with control spots ultimately simplifies the analyses of product. All R_f's are presented as the consequent two-dimensional R_X's (X,Y-coordinates), and converted to exact R_f's by the formula $(R_X - 1)/19$. Autoradiogram exposure represents radioactive dNMP's. The sum of all dNMP's equal 100%, and any individual percentage is equal to dNMP 'X'/dNMP Sum.

2.5. Institutional Review Board (IRB) human studies

This study underwent IRB review and received approval for anonymous patients' allowance of discarded samples, where no traceable characteristics were evident or allowed, with the exception of gender, HIV positivity, and active AZT use.

3. Results

Four anonymous blood samples from HIV-positive patients, all under AZT therapy, from the HIV clinic in Montefiore Medical Center, were tested for AZT–DNA analogues and adduct formation by using ^{32}P-post-labeling 2-D TLC assay. Calf thymus, and human DNA were used as control. Migration patterns of calf thymus DNA and human DNA are presented in Fig. 1. The migration patterns of four nucleotides in both species are same. Thymine glycol, which is formed through oxidative stress, is present in both species (*cis*/*trans*).

Fig. 2 shows 2-D TCL DNA autoradiograms of four patients. Adducts are present in all patients' DNA samples. It turns out that DNA perturbation was a common phenomenon in patients under AZT therapy.

Quantification of the nucleotides on autoradiograms was achieved by densitometric scanning. The scanned data were further analyzed by ImageQuant software program. The density percentages for each spot are summarized in Table 1.

Samples from patients #1 to 4 were run twice, respectively, to assess intra-assay variability. Percentages of distributed densities of four nucleotides, dAMP, dGMP, dTMP, dCMP, were used because they represented the integrity of DNA fingerprinting on autoradiograms. Intra-assay variability assessment was measured on the correlation between two 2-D TLC assays for each DNA sample. Total intraclass coefficient of correlation (r = 0.88, P = 0.0001) was obtained from four patients' DNA assays that produced

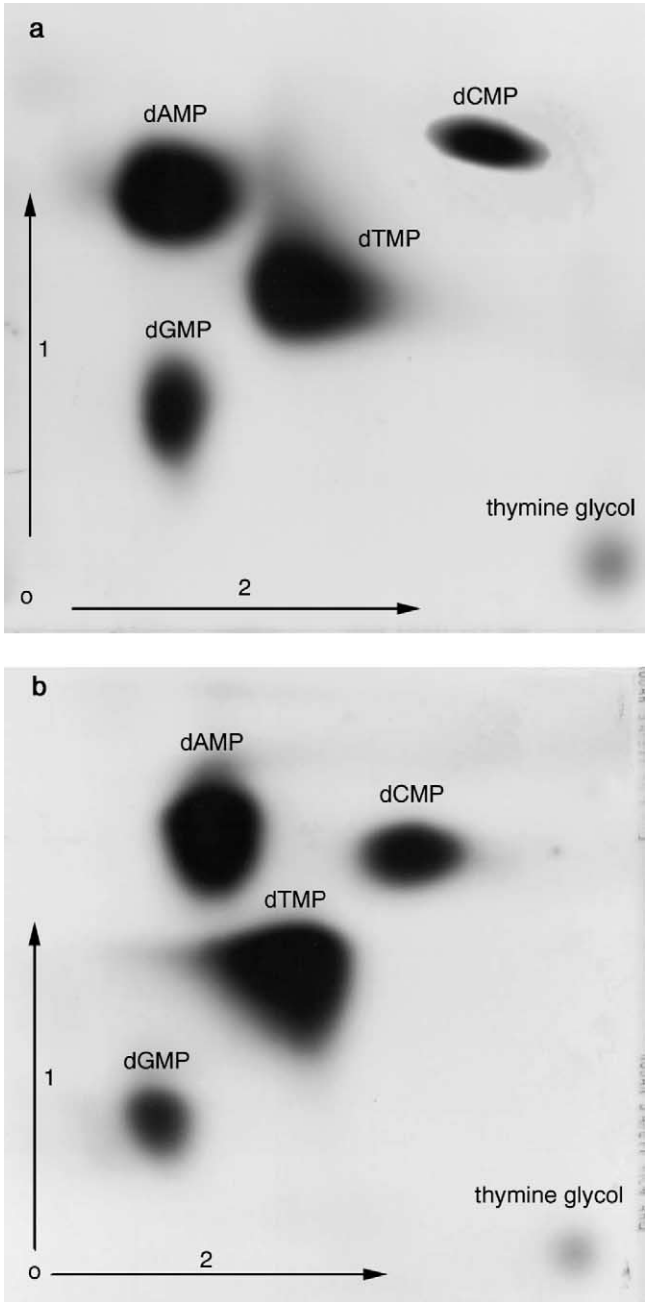

Fig. 1. Two-D TLC nucleotide migration patterns of calf thymus DNA and human leukocyte DNA. One microgram of extracted DNA labeled with ^{32}P dNTPs by nick translation, and digested with spleen phosphodiesterase II and micrococcal nuclease runs on the 2-D TLC. The dried cellulose is exposure to the Kodak XAR-5 film for 20 h. Four nucleotides from calf thymus DNA (panel a) and human leukocyte DNA (panel b) are well separated. The arrows point to the first and second migration directions. Letter 'o' marks the original sample-loading place.

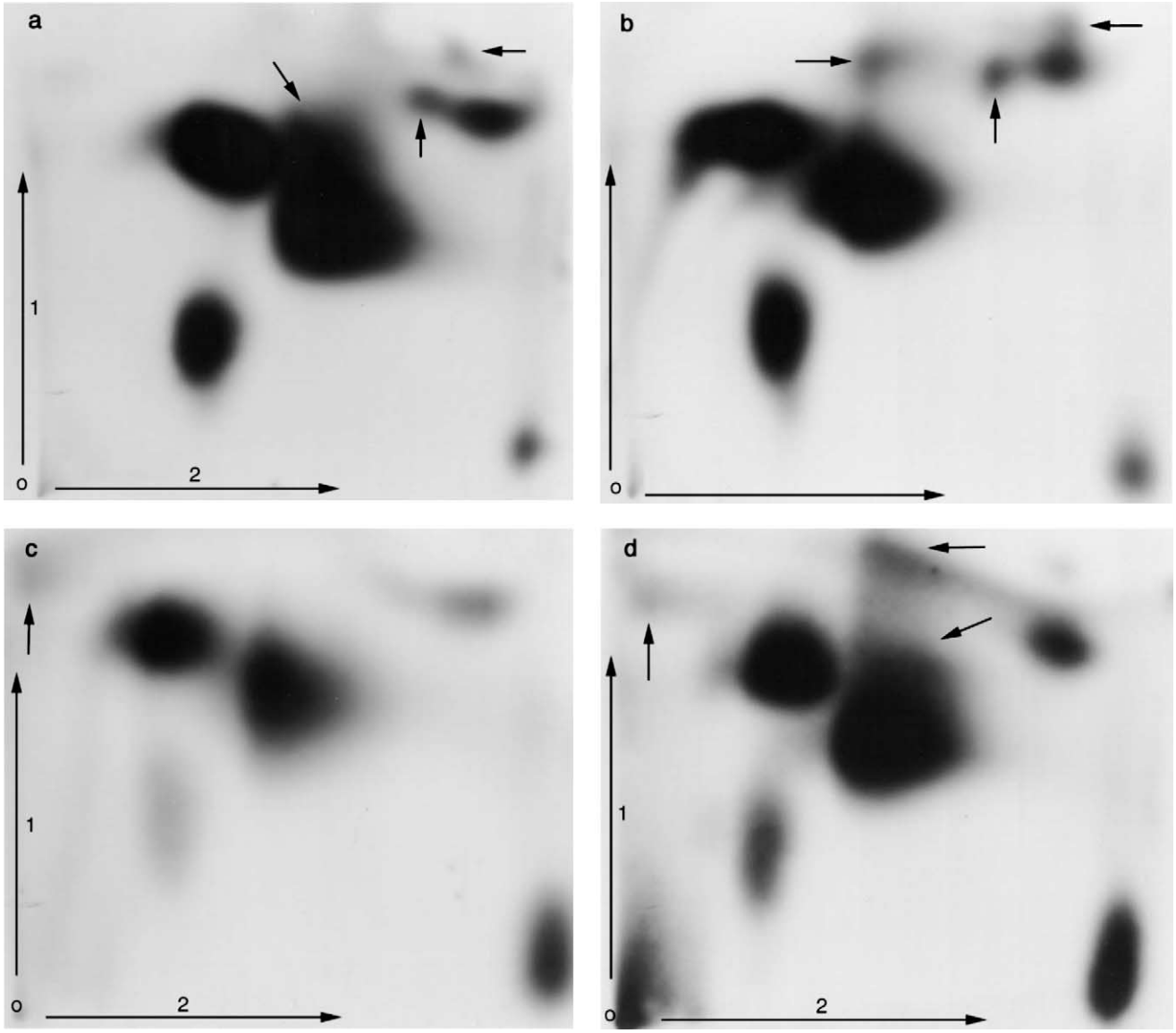

Fig. 2. Two-D TLC nucleotide migration patterns of peripheral blood leukocytes DNA from HIV positive patients under AZT therapy. Experimental condition is same as Fig. 1. Patient #1 (panel a), #2 (panel b), #4 (panel d) show three altered nucleic acid spots or adducts, respectively. Patient #3 (panel c) shows one adduct spot. Short arrows point to the unknown adducts, and long arrows point to the first and second migration directions. Letter 'o' marks the original sample-loading place.

16 nucleotide density spots summarized in Fig. 3a. Intra-class correlation on assays conducted at different days were analyzed to measure inter-assay variability. It is highly correlated with $r = 0.89$ and $P = 0.0004$ as showed in Fig. 3b.

4. Discussion

To establish an efficient assessment to measuring AZT–DNA analogue and adducts formed in patients under AIDS therapies is a necessity. AZT incorporation into

Table 1
The changes of the nucleotide components in patients with AZT therapy

	Calf	Human	Patient #l	Patient #2	Patient #3	Patient #4
dAMP (%)	36.02	33.31	30.85	11.91	29.50	20.73
dTMP (%)	35.00	36.69	37.90	44.79	63.88	44.23
dGMP (%)	16.73	12.58	15.29	24.16	2.30	8.89
dCMP (%)	12.25	17.42	9.16	2.84	3.58	8.27
Adducts (%)	0.00	0.00	6.80	16.1	0.74	17.88

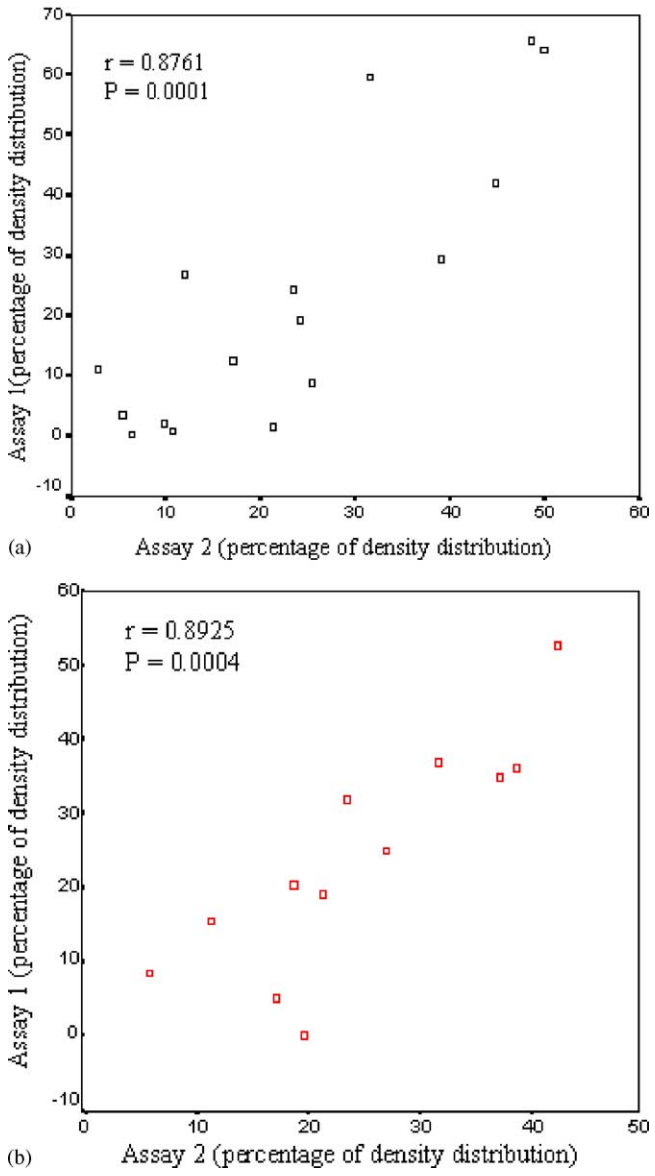

Fig. 3. Two-D TLC reliability assessment. The intra-assay variability (panel a) was obtained from the duplicated experiments in same day. The inter-assay variability (panel b) was obtained from the repeated experiments in different days. The coefficient correlation and *P* values are shown in each panel.

mammalian DNA and adducts localizing in telomeres have been documented by immunohistochemistry. Electron microscopy revealed mitochondrial damage [11]. The frequency of chromosome breaks in the AZT groups is increased [12]. Southern blot analyses indicated loss of heterozygosity, consistent with the known mechanism of AZT as a DNA chain terminator [13]. Fluorouracil doubled AZT incorporating into DNA in cells exposed to AZT [14]. AZT is a transplacental mutagen in monkey and mouse [15,16]. Incorporation of AZT into vaginal DNA epithelium may play a role in abnormal differentiation and tumorigenesis in mice [17]. AZT crosses the human placenta and becomes rapidly incorporated into DNA of placental tissue [18].

Currently, the most commonly used methods for DNA adduct measurement are immunoassays and ^{32}P-post-labeling assay. The assay is reliable and inexpensive, but with disadvantages of antibody cross-reaction and large 200 μg DNA sample requirement. The latter is more sensitive. It requires about 2–20 μg DNA as running sample. The disadvantages of ^{32}P-post-labeling assay are the unidentifiable adducts and the uncontrolled phosphorylation [19]. We developed ^{32}P-post-labeling 2-D TLC assay to detect radioactivity labeled nucleic acid components quantitatively and qualitatively [20]. This assay has been successfully reported to measure 2′-deoxyuridine-3′-monophosphate (dUMP) adduct [21], 5-bromo-2′-deoxiuridine (BUDR) adduct [22], and gliotoxin induced adduct [23] in tested DNA samples. This technique is sensitive enough to detect one adduct per 10^5 nucleotides. The 2-D TLC system will separate purines from pyrimidines of nucleic acid derivatives by taking advantage of the various mobilities of different nucleotides or exogenous molecules on the TLC cellulose. The 5′-^{32}P-radiolabeled nucleotides are analyzed qualitatively by autoradiography and quantitatively by densitometry. The advantage of the assay is very sensitive so that there is only 1 ng DNA needed. In this study, we use this assay further to detect the AZT induced host DNA-adducts in HIV positive patients who are under AIDS therapy.

The spot densities in Fig. 1, pattern of controlled calf thymus and human lymphocyte DNA, and sizes of adenine and thymine, guanine and cytosine are similar, and indicate assay reliability. The relative percentages of each nucleotide are presented in Table 1. Four leukocyte DNA specimens from AIDS patients under AZT therapy were tested by this technique with respective nucleotide base migration patterns shown in Fig. 2. Comparing to the control specimen, the varieties of unknown aberrant bases appeared in all patients' specimens. Their relative quantities are shown in Table 1. They might represent the AZT-adducts or perturbations induced by the AIDS therapy. More interestingly, the densitometry data shows that the density and size ratio between adenine and thymine are significantly changed ($P < 0.01$). It may suggest that the AZT compound might effect the adenines more than the thymines. The appearances of aberrant nucleotides in AZT treated patients in this study further provide the possibilities of cytotoxicity of AZT therapy. These aberrant dNMPs need to be explained, followed, and understood. The assay has the ability to detect these demonstrable changes.

In summary, this study demonstrates the feasibility of assaying DNA samples from AIDS patients who are under AZT or HAART therapy by ^{32}P-post-labeling 2-D TLC assay. The demonstrated nucleotide adducts might be the biomarkers contributing to the AZT cytotoxicity. The ^{32}P-post-labeling 2-D TLC assay is reproducible and reliable. Its practicality should be further determined by larger clinical trials in well-followed patient populations.

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