Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 58 (2003) 125–129
Antiviral Research
www.elsevier.com/locate/antiviral

A new sensitive cartridge-RIA method for determination of stavudine (D4T) triphosphate in human cells in vivo

Thu T. Tran [a], Brian L. Robbins [a,*], Frank H. Pinkerton, Jr. [a], Bernard Ferrua [b], Jacques Grassi [c], Arnold Fridland [a,d,1]

[a] *Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38105, USA*
[b] *Laboratoire de Pharmacologie, Faculte de Medecine de Nice, 06000 Nice, France*
[c] *CEA, Pharmacology and Immunology Unit, DSV/DRM, CEA/Saclay, 9119 Gif-sur-Yvette Cedex, France*
[d] *Department of Pharmacology, University of Tennessee, Memphis, TN 38101, USA*

Received 20 August 2001; accepted 7 October 2002

Abstract

We describe a simple and sensitive method to determine stavudine triphosphate, the active intracellular anabolite of stavudine (D4T). Quantification of D4T triphosphate was performed with a combined cartridge-radioimmunoassay (cartridge-RIA) which enabled us to measure concentrations of D4T triphosphate as low as 0.5 ng/ml, or an intracellular concentration which corresponds to 20 fmol/10^6 cells if diluted like our previously published zidovudine (ZDV) assay. The only alternate methodology at present employs liquid chromatography mass spectroscopy (LC–MS/MS). The use of the cartridge-RIA methodology provides a cost-effective alternative for the determination of in vivo cellular pharmacokinetics studies of D4T in human immunodeficiency virus (HIV)-infected persons.

Keywords: Stavudine; Nucleoside reverse transcriptase inhibitors; Intracellular triphosphates; Radioimmunoassay; Acquired immune deficiency syndrome

1. Introduction

The progress in the field of anti-viral treatment has been considerable. Clinical studies have shown that combination therapies with two or more drugs are effective treatment of human immunodeficiency virus (HIV)-infected subjects (Gulick et al., 1997; Gröschel et al., 2000; Merrill et al., 1996; Pollard, 1996). Nucleoside reverse transcriptase inhibitors (NRTI) are mainstays of current anti-viral therapies for the treatment of HIV infection (Merrill et al., 1996; Pollard, 1996). Nucleoside analogs require intracellular activation to their triphosphate derivatives, the active inhibitors of HIV reverse transcriptase (Ahluwalia et al., 1996).

A quantitative correlation between plasma drug concentration and anti-HIV activity has not been defined for these drugs, making dose adjustment based on fixed plasma levels ambiguous. Studies (Gröschel et al., 2000; Fletcher et al., 1998; Hoggard et al., 2001) have suggested that the intracellular concentrations of NRTI triphosphates are more likely to be clinically relevant than plasma drug concentrations. Studies with zidovudine (ZDV), have shown that the intracellular ZDV triphosphate (ZDV-TP) concentrations correlate with changes in anti-viral activity and immunologic changes of therapy, while no such relationship with plasma drug concentration are observed (Fletcher et al., 1998). Decrease in nucleoside analog phosphorylating activity in HIV-infected subjects with advanced disease and low CD4 cells has also been reported (Jacobsson et al., 1995). Measurement of triphosphate levels and its correlation with the viral load may allow for more effective drug therapies in HIV-infected patients (Gröschel et al., 2000; Fletcher et al., 1998).

Stavudine (D4T), a structural analog of thymidine, has become widely used as part of combination regimens in both pediatric and adult HIV-infected subjects (Ahluwalia et al., 1996; Cretton et al., 1993; Hitchcock, 1991; Kaul et al., 1995; Kline et al., 1995; Rana and Dudley, 1997). In this study, we describe a new sensitive cartridge-radioimmunoassay (cartridge-RIA) for D4T that allows for rapid and relatively easy quantitation of D4T-triphosphate (D4T-TP) in peripheral blood mononuclear cells (PBMCs).

* Corresponding author.
E-mail address: afridland@gilead.com (A. Fridland).
[1] Present address: Gilead Sciences, 344 Lakeside Drive, Foster City, CA 94404, USA.

0166-3542/02/$ – see front matter
PII: S0166-3542(02)00192-4

2. Materials and methods

2.1. Materials

[^{3}H]D4T was obtained from Moravek Biochemical Brea, CA. RPMI-1640, non-essential amino acids and fetal calf serum were obtained from BioWhittaker Baltimore, MD. Interleukin 2 was purchased from Boehringer Mannheim Indianapolis, IN and lymphocyte separation media (LSM) was obtained from Organon Teknika Corp. Durham, NC. Waters QMA and C-18 Sep Pak cartridges (360 mg) were purchased from the Waters division of Millipore Milford, MA. The reagents for D4T determination by RIA, Goat anti-rabbit precipitating complex, acetic anhydride and acid phosphatase type XA were purchased from Sigma, St. Louis, MO. The radioactive [^{3}H] labeled D4T derivative was obtained from Moravek Biochemical Brea, CA and the primary antibody was obtained from Jacques Grassi. Other fine chemicals were purchased from Sigma, St. Louis, MO, Fisher Scientific Fair Lawn, NJ, or Calbiochem, LaJolla, CA.

2.2. Preparation of stimulated PBMC cell extracts

PBMC used for the analysis of intracellular concentration of D4T-TP were isolated and stimulated with phytohemagglutinin (PHA) and IL-2 as described (Munch-Petersen and Tyrsted, 1977). The activated PBMC (20 million) were incubated for 16 h with 5, 20 or 50 μM D4T. These concentrations were used to simulate peak plasma concentrations in human subjects which can range from 5 to 20 μM depending on the dosage of D4T administered (Dudley, 1995). After incubation the cells were harvested by centrifugation at 400 × *g* for 10 min. The cell pellets were re-suspended in 1 ml of residual medium, separated by centrifugation through Nyosil oil (30 s at 14000 × *g*) and extracted with 70% methanol-15 mM Tris–HCl buffer on ice. The debris was removed by centrifugation, and the samples were stored at −20 °C until use.

2.3. Cartridge separation of D4T-TP

D4T metabolites were separated with QMA anion-exchange cartridges (Waters Co., Milford Mass.). The QMA cartridges were preconditioned with 10 ml each of 1 M potassium chloride (KCl), and 5 mM KCl. Samples from 20 million cells were diluted to 1 ml with 50 mM HEPES, pH 7.0 and loaded onto the cartridges. The cartridges were washed with 5.4 ml of 5 mM KCl followed by 15 ml of 74.5 mM KCl. The bound D4T-TP was eluted with 3.4 ml of 1 M KCl. The triphosphate fractions were acidified to pH 4.5 with 50 μl per ml of 1 M sodium acetate buffer. The phosphate groups were removed by incubation at 37 °C for 30 min with 1 U of acid phosphatase per ml (Type XA, Sigma, St. Louis, MO). The samples were then desalted using Waters Sep Pak C-18 cartridges. The C-18 cartridges (Waters Co., Milford Mass.) were preconditioned with 10 ml each of acetonitrile and water. The dephosphorylated triphosphate fractions were loaded onto the C-18 cartridges and washed with 10 ml of Millipore water to remove the salt from QMA cartridge elution. Bound D4T was eluted from the C-18 cartridges with 5 ml of acetonitrile. The samples were then evaporated to dryness in a Savant Speed Vac and dissolved with 0.2 ml of phosphate buffer (50 mM, pH 7.2).

2.4. Acetylation procedure

After elution from the cartridge, the resultant dephosphorylated D4T and standards, were acetylated using method similar to that described (Delaage et al., 1978; Harper and Brooker, 1975; Sato et al., 1982), with the following modifications. The samples (200 μl) were acetylated by adding 5 μl of 9N potassium hydroxide (KOH), vortexing, adding 5 μl acetic anhydride, vortexing 15 s, adding an additional 5 μl of acetic anhydride, vortexing and finally neutralizing with 15 μl of 9N KOH. Acetylated samples were evaporated to dryness using a Savant Speed Vac and dissolved with water to final volumes of 200 μl for sample and reference tubes, or 300 μl for non-specific binding tubes (no primary antibody will be added so 100 μl of water is substituted).

2.5. Radioimmunoassay procedure

The radioimmunoassay (RIA) was performed in duplicate in borosilicate glass tubes. One hundred microliter of anti-D4T antibody were added to 200 μl volumes of cellular D4T unknowns or standard curve from the previous step (but not in the non-specific binding tubes). The tubes were mixed and incubated for 95 min at room temperature. One hundred microliter of radioactive D4T substrate (Moravek), were added to each tube and incubated for 45 min. After incubation, 500 μl of goat anti-rabbit immunoglobulin G immunoprecipitating complex were added to all tubes except to the total count tubes (TC), mixed gently, and incubated for 30 min. The samples were centrifuged at 2000 × *g* for 30 min at 4 °C, the supernatants discarded and the tubes inverted on absorbent paper for at least 5 min. Six hundred microliters of hydrochloric acid (0.1N) were added to each tube, except the total count tubes where only 500 μl were added to the 100 μl of label already present. Aliquots of 500 μl of each sample were transferred to scintillation vials and 9 ml of scintillation cocktail were added to each tube and samples counted on a beta counter for 2 min.

3. Results

3.1. D4T-TP assay development

A comparison of the standard curves of the acetylated and unmodified D4T-RIA is shown in Fig. 1. As shown by the data, acetylation of D4T led to a 10-fold increase in sensitivity of the RIA, as indicated by a shift to the left

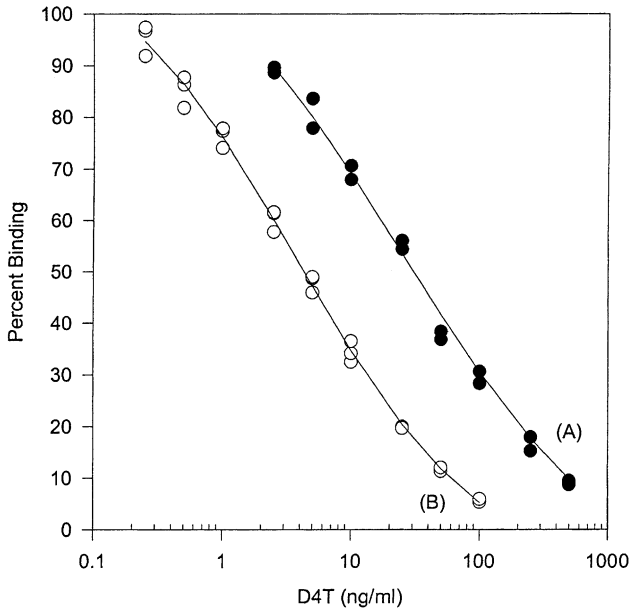

Fig. 1. Effect of acetylation on the sensitivity of the D4T RIA. Standard curves were run with ranges from 5 to 500 ng/ml with the normal RIA (A) and 0.25 to 100 ng/ml with the acetylated assay (B).

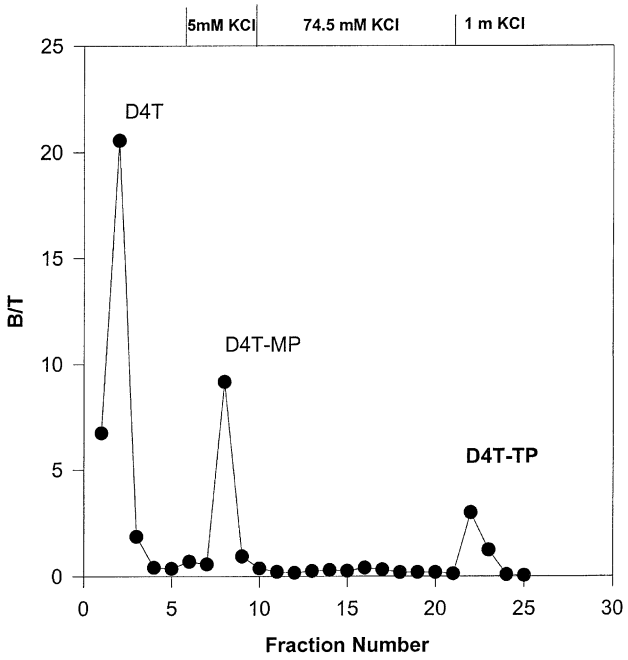

Fig. 2. The elution profile of D4T-TP, D4T-MP and D4T as separated using QMA cartridges.

of the D4T standard curve. Acetylation of the sample also decreased the limit of quantitation (LOQ) to 0.5 ng/ml as defined by having %error and %CV values of 25% or less (Shah et al., 2000).

Table 1 summarizes the variation and error of the standard curves over six assays with standards ranging from 0.25 to 100 ng/ml. The assay proved to be reliable with coefficient of variations from 7 to 25%.

The specificity of the D4T antibody was determined using a wide range of concentrations (100–10,000 ng/ml) of various anti-viral compounds including zidovudine, didanosine, zalcitabine, lamivudine and the natural nucleoside, thymidine. There was less than 0.05% cross-reactivity with all compounds except zidovudine that had a cross-reactivity of 0.25%. Moreover, other experiments indicated that sample acetylation did not significantly increase the cross-reactivity with other nucleotides (i.e. TTP).

Table 1
Standard curve statistics for the D4T assay

D4T concentration (ng/ml)	Mean calculated (ng/ml)	%Error (Δ (mean))	S.D.	%CV
0.25	0.23	−7	0.058	25
0.5	0.52	4	0.11	21
1.0	1.05	5	0.16	15
2.5	2.39	−4	0.26	11
5.0	5.08	2	0.49	10
10	10.11	1	0.67	7
25	25.39	2	2.42	10
50	49.51	−1	3.49	7
100	99.46	−1	13.61	14

Standard curve statistics were determined from all data points taken from six experiments performed over 6 days in replicates of three samples.

Separation of the phosphorylated D4T anabolites was achieved using ion-exchange cartridges procedure described previously for zidovudine (ZDV) and lamivudine (3TC; Robbins et al., 1996, 1998). Fig. 2 shows a typical elution profile for D4T, D4T-MP and D4T-TP spiked PBMC extracts, recovery of these anabolites was usually >95%. Table 2 shows both the intra-day and inter-day variation of the assay. The intra-day %CV over the four concentrations tested ranged from 6 to 37 and is reduced from 4 to 22 if outliers are excluded by the method of Dixon. The overall inter-assay (day-to-day) variability is reflected by the %CV of all the data at each concentration. This varied from 13 to 28% which is reduced to 10 to 17% if outliers are excluded by the method of Dixon. If one examines the means exclusively, the variability was 5 to 15% as indicated in Table 2.

3.2. Quantification of D4T-TP in PBMC extracts from volunteers

The number of PBMCs recovered from blood of patients varies considerably between different patients and differed between time samples. As shown by the results in Table 3, with the current assay we were able to measure D4T-TP concentrations in PBMC exposed to three different D4T concentrations ranging from 5 to 50 μM with only 10 million cells. The variability in the recovery of D4T-TP over this concentration range was estimated at 2.4–11.6.

Pharmacokinetic models frequently deal with extracellular compartments therefore plasma determination of the parent drug concentration is of interest (Horton et al., 1995). Moreover, the sensitivity of this RIA allows the

Table 2
Variability of the D4T-TP assay

Day	0.50		5.0		10.0		50.0	
	All data	Dixon	All data	Dixon	All data	Dixon	All data	Dixon
1	0.54 ± 0.20	0.60 ± 0.12	4.32 ± 0.64	4.32 ± 0.64	9.07 ± 1.37	8.61 ± 0.86	44.97 ± 7.01	47.74 ± 1.99
%CV	37	20	15	15	15	10	16	4
2	0.50 ± 0.12	0.56 ± 0.03	3.97 ± 0.85	3.97 ± 0.85	8.86 ± 0.10	9.23 ± 0.47	40.39 ± 6.1	40.39 ± 6.1
%CV	24	5	21	21	11	5	15	15
3	0.45 ± 0.14	0.46 ± 0.03	3.84 ± 0.35	3.84 ± 0.35	7.48 ± 0.64	7.71 ± 0.32	38.68 ± 3.53	38.68 ± 3.53
%CV	24	6	9	9	9	4	9	9
4	0.38 ± 0.07	0.38 ± 0.07	4.00 ± 0.39	4.00 ± 0.39	9.02 ± 1.24	9.49 ± 0.55	41.71 ± 3.47	43.01 ± 1.56
%CV	18	18	10	10	14	6	8	4
5	0.60 ± 0.10	0.60 ± 0.10	3.98 ± 0.30	3.98 ± 0.30	7.89 ± 0.73	7.89 ± 0.73	36.85 ± 2.99	36.85 ± 2.99
%CV	17	17	8	8	9	9	8	8
6	0.51 ± 0.11	0.51 ± 0.11	4.29 ± 0.54	4.29 ± 0.54	8.08 ± 0.51	8.08 ± 0.51	38.02 ± 3.03	38.02 ± 3.03
%CV	22	22	13	13	6	6	8	8
Mean ± S.D. all data	0.50 ± 0.14	0.52 ± 0.12	4.07 ± 0.54	4.07 ± 0.54	8.40 ± 1.09	8.47 ± 0.87	40.10 ± 5.08	40.51 ± 4.91
%CV	28	23	13	13	13	10	13	12
Stats of means	0.50 ± 0.08	0.52 ± 0.09	4.07 ± 0.19	4.07 ± 0.19	8.40 ± 0.67	8.50 ± 0.73	40.10 ± 2.94	40.78 ± 4.02
%CV	15	17	5	5	8	9	7	10

Table 3
Concentration of D4T-TP in stimulated PBMC determined by cartridge-RIA

	D4T-TP pmol/10^6	Range pmol/10^6	%CV
5 μM	1.10	0.03	11.6
20 μM	2.85	0.07	2.4
50 μM	4.25	0.20	8.7

Stimulated PBMC were incubated for 16 h with the indicated concentration of D4T. The cells were extracted and D4T-TP concentration determined by cartridge-RIA. Data are the means of two experiments run with 10 or 20 million cells.

Table 4
Recovery of D4T in plasma

Plasma spike with 200 ng/ml	[Target] ng/ml	Mean (ng/ml)	Range (ng/ml)	%Difference from target
1:50	4.0	4.52	0.84	13
1:100	2.0	1.98	0.08	−1

Duplicate plasma samples were spiked with 200 ng/ml of D4T diluted 1:50 or 1:100 and the concentration of D4T determined by RIA.

measurement of very low concentrations of D4T in plasma (Table 4), permitting the use of a small volume of plasma as might be obtained from pediatric samples.

4. Discussion

As described previously, phosphorylated metabolites can be rapidly separated using an ion-exchange cartridge, instead of HPLC. This cartridge-RIA was developed for the quantification of intracellular D4T-TP. Acetylation of samples containing D4T prior to the RIA increase the sensitivity of the D4T-RIA about 10-fold. Sensitivity of the RIA is increased because acetylation of D4T results in a product structurally more similar to the BSA-5′-hemisuccinate-D4T immunogen than the unmodified D4T thus allowing a more effective interaction (Ferrua et al., 1994; Zhou et al., 1996). This acetylation procedure can be applied to any RIA where the primary antibodies use the 5′-succinyl nucleoside spacer in the production of the immunogen (Kaul et al., 1996).

The LOQ of the RIA with acetylation was estimated to be 0.5 ng/ml (Table 1) which corresponds to an intracellular concentration of 20 fmol/10^6 cells (Robbins et al., 1996). Current LC–MS/MS procedures show LOQ values of 70 and 100 fmol/10^6 cells, respectively (Becher et al., 2002) and (Rodriguez et al., 2000). This RIA was developed as viable cost-effective alternative to the use of LC–MS/MS spectrometers with comparable sensitivity. Additionally, the increased sensitivity achieved with the cartridge-RIA assay can be applied to measure plasma D4T in very small volumes. Crude plasma samples were filtered through a micro-concentrator device to eliminate most of the protein. In addition this device could minimize HIV contamination since heat inactivation of the sample (1 h, 56 °C) breaks down D4T-TP samples (Rana and Dudley, 1997) and is therefore inappropriate for use in the analysis. The resulting filtrate (100 μl) is diluted 1:50 to 1:100 with sample buffer before analysis, which reduces matrix effects and the amount of plasma required.

In summary, an analytical method combining anion exchange and RIA was developed for the quantitation of intracellular D4T-TP in human PBMCs. Although this validation was performed primarily for the triphosphate, this technique may be applied to measure D4T mono- and diphosphates, since the anion cartridge method resolves the mono-, di- and triphosphate analytes. Analysis of the

different anabolites in HIV-infected patient PBMCs should lead to a better understanding of the kinetics of drug activation in patients and establish potential clinical determinants of therapeutic activity of anti-viral NRTI (Balzarini, 2000).

Acknowledgements

This work was supported in part by NIAID 2 R21 A127652-15 and the Cancer Center Core grant no. P30 CA21765 from the National Institutes of Health, and the American Lebanese Syrian Associated Charities. The authors would like to acknowledge Dr. Agrofoglio, ICOA-CNRS, University of Orleans, France for the synthesis of D4T.

References

Ahluwalia, G.S., Gao, W.-Y., Mitsuya, H., Johns, D.G., 1996. 2′, 3′-Didehydro-3′-deoxythymidine: regulation of its metabolic by modulators of thymidine-5′-triphosphate biosynthesis. Mol. Pharmacol. 50, 160–165.

Balzarini, J., 2000. Effect of antimetabolite drugs of nucleotide metabolism on anti-human immunodeficiency virus activity of nucleoside reverse transcriptase inhibitors. Pharmacol. Ther. 87, 175–187.

Becher, F., Pruvost, A., Goujard, C., Guerreiro, C., Delfraissy, J.-F., Grassi, J., Benech, H., 2002. Improved method for the simultaneous determination of D4T, 3TC and ddI intracellular phosphorylated anabolites in human peripheral-blood lmononuclear cells using high-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 16, 555–565.

Cretton, E.M., Zhu, Z., Kidd, L.B., McClure, H.M., Kaul, S., Hitchcock, M.J.M., Sommadossi, J.P., 1993. In vitro and in vivo disposition and metabolism of 3′-deoxy-2′,3′-didehydrothymidine. Antimicrob. Agent Chemother. 37 (9), 1816–1825.

Delaage, M.A., Roux, D., Cailla, H.L., 1978. In: Falco, G., Paoletti, R. (Eds.), Molecular Biology and Pharmacology of Cyclic Nucleotides. Elsevier/North-Holland, Amsterdam, pp. 155–171.

Dudley, M.N., 1995. Clinical pharmacokinetics of nucleoside antiretroviral agents. J. Infect. Dis. 171 (Suppl. 2), S100–S112.

Ferrua, B., Tran, T.T., Quaranta, J.F., Kubar, J., Roptin, C., Condom, R., Durant, J., Guedj, R., 1994. Measurement of the anti-HIV agent 2′,3′-didehydro-2′,3′-dideothymidine (D4T) by competitive ELISA. J. Immunol. Methods 176, 103–110.

Fletcher, C.V., Acosta, E.P., Henry, K., Page, L.M., Gross, C.R., Kawle, S.P., Remmel, R.P., Erice, A., Bafour, H.H., 1998. Concentration-controlled zidovudine therapy. Clin. Pharmacol. Ther. 64, 331–338.

Gröschel, B., Miller, V., Doerr, H.W., Cinatl Jr., J., 2000. Activity of cellular thymidine kinase 1 in PBMC of HIV-1-infected patients: novel therapy marker. Infection 28, 209–213.

Gulick, R.M., Mellors, J.W., Havlir, D., Eron, J.J., Gonzalez, C., McMahon, D., Richman, D.D., Valentine, F.T., Jonas, L., Meibohm, A., Emini, E.A., Chadakewitz, J.A., 1997. Control trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell count of 200 per cubic millimeter or less. New Eng. J. Med. 337, 734–739.

Harper, J.F., Brooker, G., 1975. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′-*O*-acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucleotide Res. 1, 207–218.

Hitchcock, M.J.M., 1991. 2′,3′-Didehydro-2′,3′-dideoxythymidine (D4T), an anti-HIV agent. Antiviral Chem. Chemother. 2 (3), 125–132.

Hoggard, P.G., Sales, S.D., Phiboobanakit, D., Lloyd, J., Maher, B.A., Khoo, S.H., Wilkins, E., Carey, P., Hart, C.A., Back, D., 2001. Influence of prior exposure to zidovudine on stavudine phosphorylation in vivo and ex vivo. Antimicrob. Agents Chemother. 45 (2), 577–582.

Horton, C.M., Dudley, M.N., Kaul, S., Mayer, K.H., Squires, K., Dunkle, L., Anderson, R., 1995. Population pharmacokinetics of Stavudine (D4T) in patients with AIDS or advanced AIDS-related complex. Antimicrob. Agents Chemother. 39 (10), 2309–2315.

Jacobsson, B., Britton, S., He, Q., Karlsson, A., Eriksson, S., 1995. Decreased thymidine kinase levels in peripheral blood cells from HIV-seropositive individuals: implications for zidovudine metabolism. AIDS Hum. Retroviruses 11 (7), 805–811.

Kaul, S., Mummaneni, V., Barbhaiya, R., 1995. Dose proportionality of stavudine in HIV seropositive asymptomatic subjects: application to bioequivalence assessment of various capsule formulations. Biopharm. Drug Disp. 16, 125–136.

Kaul, S., Stouffer, B., Mummaneni, V., Turabi, N., Mantha, S., Jayatilak, P., Barbhaiya, R., 1996. Specific radioimmunoassay for the measurement of stavudine in human plasma and urine. J. Pharm. Biomed. Anal. 15, 165–174.

Kline, M.W., Dunkle, L.M., Church, J.A., Goldsmith, J.C., Harris, A.T., Federici, M.E., Schultze, M.E., Woods, L., Coewen, D.F., Kaul, S., 1995. A phase I/II evaluation of stavudine (D4T) in children with human immunodeficiency virus infection. Pediatrics 96 (2), 247–252.

Merrill, D.P., Moonis, M., Chou, T.C., Hirsh, M.S., 1996. Lamivudine or stavudine in two- and three-drug combinations against human immunodeficiency virus type 1 replication in vitro. J. Infect. Dis. 173, 355–364.

Munch-Petersen, B., Tyrsted, G., 1977. Induction of thymidine kinases in phytohaemagglutinnin-stimulated human lymphocytes. Biochim. Biophys. Acta 478, 364–375.

Pollard, R.B., 1996. D4T nucleoside combination for HIV. Antiviral. Res. 29, 101–104.

Rana, K.Z., Dudley, M.N., 1997. Clinical pharmacokinetics of stavudine. Clin. Pharmacokinet. 33 (4), 276–284.

Robbins, B.L., Waibel, B.H., Fridland, A., 1996. Quantitation of Intracellular zidovudine phosphates by use of combined cartridge-radioimmunoassay methodology. Antimicrob. Agents Chemother. 40 (11), 2651–2654.

Robbins, B.L., Tran, T.T., Pinkerton Jr., F.H., Akeb, F., Guedj, R., Grassi, J., Lancaster, D., Fridland, A., 1998. Development of a new cartridge radioimmunoassay for the determination of intracellular levels of lamivudine triphosphate in peripheral blood mononuclear cells of human immunodefiency virus infected patients. Antimicrob. Agents Chemother. 42 (10), 2656–2660.

Rodriguez, J.F., Rodriguez, J.L., Santana, J., Garcia, H., Rosario, O., 2000. Simultaneous quantitation of intracellular zidovudine and lamivudine triphosphates in human immunodeficiency virus-infected individuals. Antimicrob. Agents Chemother. 44 (11), 3097–3100.

Sato, T., Kuninaka, A., Yoshino, H., 1982. A practically sensitive radioimmunoassay for cyclic CMP by 2′-*O*-acetylation. Anal. Biochem. 123, 208–218.

Shah, V.P., Midha, K.K., Findlay, J.W.A., Hill, H.M., Hulse, J.D., McGilveray, I.J., McKay, G., Miller, K.J., Patnaik, R.N., Powell, M.L., Tonelli, A., Viswanathan, C.T., Yacobi, A., 2000. Bioanalytical method validation: a revisit with a decade of progress. Pharm. Res. 17, 1551–1557.

Zhou, X.-J., Chakboub, H., Ferrua, B., Moravek, J., Guedj, R., Sommadossi, J.-P., 1996. Radioimmunoassay for quantitation of 2′,3′-didehydro-3′-deoxythymidine (D4T) in human plasma. Antimicrob. Agents Chemother. 40 (6), 1472–1475.