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Short communication

The stability of intracellular zidovudine and its anabolites in extracts of peripheral blood mononuclear cells (PBMCs)

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1. Introduction

Zidovudine (ZDV, 3′-azido-2,3′-dideoxythymidine, AZT) is one of the antiretroviral drugs use in combination therapy for HIV infection [1,2]. Attempts to establish a correlation between antiviral activity and/or toxicity of the parent ZDV and its plasma pharmacokinetics have not been successful [3]. It is known that the active metabolite, which inhibits reverse transcriptase is ZDV-triphosphate formed inside the cells [4]. Therefore, it has been postulated that intracellular triphosphate concentrations may be a better predictor of antiviral response. Intracellular phosphorylation of ZDV has been well documented, and ZDV-TP inhibits HIV reverse transcriptase, by blocking chain elongation as it is incorporated into viral DNA [4]. For clinical studies, intracellular phosphorylated anabolites are extracted from the peripheral blood mononuclear cells (PBMCs), by adding 60% v/v methanol buffered to pH 7.2 with Tris in water and stored at either −20 or −70°C until quantitative analysis [5]. The stability of these anabolites in methanolic extracts of the PBMCs at either −20 or −70°C remains unknown. Therefore, it is desirable to investigate the stability of ZDV and its phosphates in the extract of PBMCs during frozen storage up to 6 months. Analytical methodologies are available which permit the quantitation of ZDV and its anabolites in PBMCs [5–11]. Among these the use of quartenary methyl amine (QMA) extraction cartridges in place of HPLC for separation allows the quantitative determination of ZDV and its anabolites to be faster and simpler. The details described here are a modification of a reported method [9] using known amounts of radiolabelled ZDV and its metabolites (tritiated on the methyl group, Fig. 1). After spiking into the extract of PBMCs (60% methanol in water buffered to pH 7.2 with Tris) it is stored at −70 and −20°C for 2 weeks, 1, 3 and 6 months. After this time spiked

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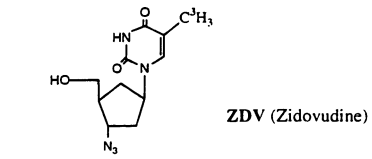
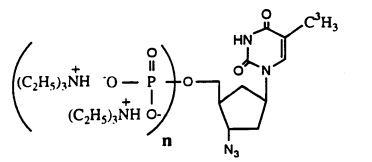
samples are separated on individual QMA cartridges, and sequentially eluted with increasing ionic strength of KCl solution. Before undertaking a clinical trial, it was essential to gather stability data of the nucleotides, since real time analysis of samples is impractical.

2. Materials and methods

2.1. Materials

All chemicals were reagent-grade unless otherwise noted. Methanol, scintillation cocktail, potassium chloride and sodium acetate were obtained from Fisher (Fair Lawn, NJ, USA). Radiolabelled ZDV and ZDV-phosphates (^3H) were purchased from Moravek Biochemical (Brea, CA). Type XA acid phosphatase was from Sigma Chemical Co. (St. Louis, MO). Phosphate buffered saline (PBS), RPMI-medium and fetal bovine serum were purchased from the Cell Culture Facility, University of California, San Francisco, CA, Sep-Pak QMA anion-exchange cartridge from Waters Co. (Milford, MA) and C18 solid phase extraction-cartridges 3 cc/500 mg from Varian (Harbor City, CA). Buffy coat pre-





n = 1, ZDV-MP
n = 2, ZDV-DP
n = 3, ZDV-TP
as triethyl ammonium salt

$^3H_3$ is the tritiated hydrogens on the methyl group of ZDV and ZDV-phosphates

Fig. 1. Structure formulas of radiolabelled ZDV and ZDV-phosphates.

separated PBMC was purchased from the Irwin Memorial Blood Bank (San Francisco, CA). Ficoll-Paque was purchased from Pharmacia Biotechnology Inc. (Piscataway, NJ).

2.2. Sample preparation

PBMCs from buffy coat were isolated and counted [5]. Drug free cells (blank) were extracted by adding 60% methanol in aqueous Tris buffer (pH 7.2) and standing at −20°C overnight [7,8]. Blank cell extract in 60% (v/v) methanol in Tris buffer was stored at −70°C until the day of spiking ZDV and ZDV-phosphates into the cell extract for storage study.

Two different concentrations of ZDV and ZDV-phosphates were selected, based on the results obtained from the treated patients for the study, aliquotted and kept at −70°C until use [12]. The extract of 2×10^6 cells were spiked in the aliquotted ZDV and ZDV-phosphate samples (in triplicates) before the 2 week, 1, 3 and 6 month periods of the study time. At the beginning of the study, the two selected concentrations of ZDV and ZDV-phosphates were separated on the individual cartridges (ZDV, 0.067 and 0.033; ZDV-MP, 0.67 and 0.0835; ZDV-DP, 0.067 and 0.011; ZDV-TP, 0.067 and 0.0167 pmol/10^6 cells) and recorded as week 0 to compare with that of week 2, month 1, 3 and 6 results.

2.3. QMA cartridge separation

The QMA cartridges were preconditioned with 10 ml of 1 M KCl solution followed by a wash of 10 ml of 5 mM KCl solution at a flowrate of 1 ml/min, using a vacuum manifold. Samples of cell extract containing ZDV and its anabolites were loaded onto the cartridges and with the effluent 8×500 μl aliquots that contained ZDV was collected. Sample tubes were thoroughly washed with 500 μl aliquots of 5 mM KCl up to a total volume of 4 ml. ZDV-MP was then eluted with 7 ml of 60 mM KCl, followed by ZDV-DP which was eluted with 11 ml of 74.5 mM KCl. The cartridges were prevented from drying during the elution of ZDV, ZDV-MP and ZDV-DP. Finally ZDV-TP was washed out with 3 to 4 ml of 1 M KCl.

2.4. Dephosphorylation and sample cleanup

ZDV-nucleotides are dephosphorylated to parent ZDV by acid phosphatase (Type XA) and the parent ZDV from the digested samples was cleaned up with Varian C-18 cartridges [9].

The residues were suspended in 500 μl of distilled water and the radioactivity in 450 μl aliquots was determined. Two different concentrations of ZDV and ZDV-phosphates, which did not undergo QMA and the solid phase extraction, were also reconstituted to 500 and 450 μl aliquots. They were mixed with scintillation cocktail for counting and these counts were considered to represent 100% recovery.

3. Results

The half life of ^{3}H is 12.3 years and hence the radioactive decay for 6 months was assumed to be negligible. Therefore, the radioactivity content of tritiated ZDV, counted as disintegrations per minute (dis/min) was directly used as a quantitative measurement, for the storage stability study of ZDV and ZDV-phosphates in the extracts of PBMCs.

3.1. Recovery of ZDV and ZDV-phosphates

Extraction recovery data, as well as reproducibility of recovery (%RSD), from extracts of PBMCs supplemented with radiolabelled ZDV, ZDV-MP, ZDV-DP and ZDV-TP are 93 ± 7.0, 91 ± 1.2, 92 ± 2.8 and 98 ± 3.1, respectively, with %RSD 7, 1, 3 and 3, correspondingly (means of five experiments).

3.2. Precision and accuracy

Interday %CV was 10, 2, 3, and 3 for ZDV, ZDV-MP, ZDV-DP and ZDV-TP, respectively, at 0.1332, 1.332, 0.1332, and 0.1336 pmol spiked extracts of two million cells ($n = 5$), with mean total activity $\pm$ S.D. (dis/min) of 616 ± 62, 7979 ± 150, 966 ± 25, and 726 ± 20, correspondingly. Intraday %CV was 5, 5, 1 and 2 for ZDV, ZDV-MP, ZDV-DP and ZDV-TP, respectively, at the same amount as interday samples with mean total activity $\pm$ S.D. (dis/min) of 617 ± 32, 8101 ± 411, 936 ± 12 and 720 ± 13, correspondingly. The radioactivity counting for the blank is less than 50 dis/min and the lower limit of quantitation based on twice background was estimated in radiolabelled ZDV (specific activity 11.5 Ci/mmol, 1.0 mCi/ml) is 0.0023 ng/ml (450 μl) which corresponds to 130 dis/min.

3.3. Interferences

The background caused by PBMCs were tested with ZDV and ZDV-phosphate free cells of 2×10^6, and separated for ZDV and ZDV-phosphates the same way as study samples and counted for radioactivity. Radioactivity of the background was below the limit of detection and therefore considered negligible.

3.4. Freeze–thaw stability of ZDV-phosphates

Known amounts of radiolabelled (^{3}H) ZDV-DP and ZDV-TP were freeze–thawed several times and separated by previously described HPLC methods [5], and compared with that of freshly prepared solutions. 85 and 35% of degradation of ZDV-TP and ZDV-DP were observed, respectively.

4. Discussion

ZDV and ZDV-phosphates were spiked individually into the extract of 2×10^6 cells, (in 60% methanol in Tris buffer) and therefore any deterioration could be observed during the storage. Extract of 2×10^6 cells were substantially enough for our study because the radioactive counting corresponding to the amounts of the tritiated ZDV and ZDV-phosphates spiked are high enough to quantify. Since the radioisotopic counts of blank cell extracts are approximately 50 dis/min, any value above this would be measurable in terms of concentration.

The ZDV-DP and ZDV-TP are found to be unstable on repeated freeze–thawing. We found by both QMA cartridge and reverse phase HPLC

Table 1
Stability of ZDV and ZDV-phosphates in the cell extract of PBMCs at −20°C[a]

Concentration 1 (pmol/2 × 10^6 cells)	**ZDV** 0.1332	**ZDV-MP** 1.332		**ZDV-DP** 0.1332			**ZDV-TP** 0.1336			
Time	**ZDV**	ZDV	**ZDV-MP**	ZDV	ZDV-MP	**ZDV-DP**	ZDV	ZDV-MP	DP	**ZDV-TP**
	Total counts (dis/min)									
Week 0	610 ± 13	114	7975 ± 88	42	110	985 ± 9	8	40	67	720 ± 21
Week 2	628 ± 11	289	8085 ± 130	43	174	931 ± 26	39	45	77	788 ± 11
% Change in total counts	3		1			−5				9
Month 1	583 ± 42	562	7848 ± 146	66	299	832 ± 43	83	80	11	637 ± 122
% Change in total counts	−4		−2			−16				−12
Month 3	511 ± 24	495	7666 ± 143	56	469	646 ± 16	40	63	87	703 ± 34
% Change in total counts	−16		−4			−34				−2
Month 6	502 ± 13	969	7405 ± 131	88	635	409 ± 15	41	108	11	681 ± 59
% Change in total counts	−18		−7			−58				−5
Concentration 2 (pmol/2 × 10^6 cells)	**ZDV** 0.066	**ZDV-MP** 0.1665		**ZDV-DP** 0.0222			**ZDV-TP** 0.0334			
Time	**ZDV**	ZDV	**ZDV-MP**	ZDV	ZDV-MP	**ZDV-DP**	ZDV	ZDV-MP	DP	**ZDV-TP**
	Total counts (dis/min)									
Week 0	337 ± 11	55	688 ± 8	–	–	205 ± 11	–	–	–	236 ± 4
Week 2	348 ± 13	61	685 ± 25	37	61	194 ± 20	38	39	44	241 ± 10
% Change in total counts	3		0			−5				2
Month 1	336 ± 24	79	646 ± 9	38	89	187 ± 24	74	58	57	146 ± 8
% Change in total counts	0		−6			−9				−38
Month 3	346 ± 8	76	639 ± 24	43	104	162 ± 11	34	45	52	213 ± 12
% Change in total counts	3		−7			−21				−10
Month 6	199 ± 24	101	549 ± 387	45	151	108 ± 4	41	54	52	197 ± 9
% Change in total counts	−41		−20			−47				−17

[a] Each value is mean of three. Data in bold face show the amounts of ZDV-MP, ZDV-DP and ZDV-TP (with S.D.) obtained at indicated storage time; whereas, the light face represents that of the corresponding lower anabolites. Negative sign denotes the loss.

Table 2
Stability of ZDV and ZDV phosphates in the cell extract of PBMCs at −70°C[a]

Concentration 1 (pmol/2 × 10^6 cells)	**ZDV** 0.1332	**ZDV-MP** 1.332		**ZDV-DP** 0.1332			**ZDV-TP** 0.1336			
Time	**ZDV**	ZDV	**ZDV-MP**	ZDV	ZDV-MP	**ZDV-DP**	ZDV	ZDV-MP	DP	**ZDV-TP**
	Total counts (dis/min)									
Week 0	610 ± 13	114	7975 ± 88	42	110	985 ± 9	38	40	67	720 ± 21
Week 2	700 ± 13	309	8144 ± 99	45	103	998 ± 13	36	39	82	731 ± 11
Month 1	637 ± 13	159	8070 ± 479	36	121	952 ± 33	37	45	102	697 ± 13
Month 3	527 ± 34	96	7748 ± 721	40	89	957 ± 46	35	36	74	752 ± 64
Month 6	605 ± 37	99	7956 ± 463	38	97	937 ± 16	35	33	85	732 ± 6
Mean (week 0 to month 6)	**616**	155	**7979**	40	104	**966**	36	39	82	**726**
S.D.	62	89	150	3	12	25	1	5	13	20
%RSD	**10**	58	**2**	9	12	**3**	4	12	16	**3**
Concentration 2 (pmol/2 × 10^6 cells)	**ZDV** 0.066	**ZDV-MP** 0.1665		**ZDV-DP** 0.022			**ZDV-TP** 0.033			
Time	**ZDV**	ZDV	**ZDV-MP**	ZDV	ZDV-MP	**ZDV-DP**	ZDV	ZDV-MP	DP	**ZDV-TP**
	Total counts (dis/min)									
Week 0	337 ± 11	55	688 ± 8	–	–	205 ± 11	–	–	–	236 ± 4
Week 2	397 ± 8	57	689 ± 9	38	49	208 ± 8	35	38	37	222 ± 6
Month 1	359 ± 25	47	684 ± 34	39	63	202 ± 4	38	35	53	207 ± 18
Month 3	319 ± 21	45	675 ± 16	32	44	213 ± 17	39	32	45	228 ± 5
Month 6	358 ± 2	41	664 ± 52	36	47	199 ± 15	35	36	47	220 ± 8
Mean (week 0 to month 6)	**354**	49	**680**	36	51	**205**	37	35	46	**223**
S.D.	29	7	11	3	8	5	2	3	7	11
%RSD	**8**	14	**2**	9	17	**2**	6	7	15	**5**

[a] Each value is mean of three. Data in bold face show the amounts of ZDV-MP, ZDV-DP and ZDV-TP (with S.D.) obtained at indicated storage time; whereas, the light face represents that of the corresponding lower anabolites.

methods that our DP and TP were found to be less than 50% pure, after a few freeze–thaw cycles. The same degradation with (^{3}H) ZDV was also found by others [10]. Therefore, it was necessary to find the percent purity of the ZDV-phosphates at the beginning of the study and consider them as week 0 results. The percent purity of ZDV and ZDV-phosphates in the previously aliquotted stock solutions were done by separating them individually on QMA cartridges. Any degraded ZDV, ZDV-MP and ZDV-DP from ZDV-MP, ZDV-DP and ZDV-TP were quantified subsequently. Freeze–thaw cycles were avoided by aliquotting the required amount of ZDV and ZDV-phosphate samples prior to the study and stored at −70°C.

QMA chloride is an anion exchange matrix where ZDV-phosphates adsorb to a different extent. Accordingly, ZDV and ZDV-phosphates can be eluted from QMA columns in a stepwise manner by increasing the ionic strength of the potassium chloride eluant from 5 mM to 1.0 M. ZDV elutes when the sample is loaded onto a cartridge, as ZDV does not possess any ionic group to adsorb onto QMA. ZDV-MP, ZDV-DP and ZDV-TP adsorbed onto the QMA with increasing order of strength and therefore ionic strength of the KCl solution is gradually increased from 60 mM to 1 M to elute these phosphates, respectively. ZDV and ZDV-phosphates were spiked individually into cell extract (60% methanol buffered with Tris pH 7.2) before 2 weeks, 1, 3 and 6 months time of storage. Spiked samples were separated on individual QMA cartridges. ZDV-MP samples were analysed for ZDV and ZDV-MP; ZDV-DP samples were analysed for ZDV, ZDV-MP and ZDV-DP; ZDV-TP samples were analysed for ZDV, ZDV-MP, ZDV-DP and ZDV-TP (Tables 1 and 2). Dephosphorylated ZDV-phosphates changed to parent ZDV by acid phosphatase. The QMA separation step was skipped in the case of the samples spiked with ZDV in the cell extract (of 60% methanol buffered with Tris) and directly followed the cleanup on C-18 cartridges after the removal of methanol. The presence of methanol in the samples does not affect the elution pattern of the anion exchange cartridge (QMA) and thereby permits the direct sample application, unlike any conventional HPLC methodologies, where the samples are dried completely and reconstituted with the mobile phase. Stability of ZDV-phosphates are unknown during the drying process for HPLC methods. This is avoided in the QMA method described above.

Radioactivity content of each sample was measured by liquid scintillation counting on a Beckman instrument. Each sample was counted for 5 min. All counts were converted to dis/min by automatic quench correction. Samples containing radioactivity of less than the background were considered to be below the limit of quantitation and considered to contain zero amount of radiolabelled ZDV and ZDV-phosphates. At −70°C, ZDV and ZDV-phosphates were found to be stable up to 6 months in the PBMCs extract with a %CV ranging from 2 to 10. However, at −20°C, ZDV-phosphates are stable for 1 month. Substantial degradation of 34 and 58% was observed in the case of ZDV-DP to ZDV-MP after 3 and 6 months storage, respectively. The 41% loss found in the case of ZDV (Table 2, concentration 2) after 6 months is due to the incomplete removal of methanol prior to the solid phase extraction on the C-18 cartridge. The presence of methanol in the sample washed out some of the ZDV during the solid phase extraction. ZDV and ZDV-phosphates were found to be stable up to 6 months at −70°C in the cell extract with 60% methanol buffered with Tris pH 7.2.

In conclusion, this study shows that −70°C is required for proper storage of ZDV-phosphates.

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